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(54) Title: GENE ENCODING NADE, P75<sup>NTR</sup>-ASSOCIATED CELL DEATH EXECUTOR AND USES THEREOF

(57) Abstract: This invention provides an isolated nucleic molecule encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor, and a purified version of said polypeptide capable of binding a p75<sup>NTR</sup> receptor. This invention provides a method of producing a purified polypeptide capable of binding a p75<sup>NTR</sup> receptor. This invention provides an antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to an mRNA molecule encoding the above described polypeptide. This invention provides a method producing a polypeptide capable of binding p75<sup>NTR</sup> receptor into a suitable vector. This invention provides a method of inducing apoptosis, a method of determining physiological effects, a method for identifying an apoptosis inducing or inhibiting compound, a method for screening cDNA libraries of said polypeptide, a method to induce caspase-2 and caspase-3 activity to cleave poly (ADP-ribose) polymerase and fragment nuclear DNA in a cell, a method to inhibit NF-kB activation in a cell, a method to detect a neurodegenerative disease, a method of producing the isolated human HGR74 protein into a suitable vector, a pharmaceutical composition comprising a purified polypeptide capable of binding a p75<sup>NTR</sup> receptor and a pharmaceutically acceptable carrier and a method of identifying a compound which is an apoptosis inhibitor.

WO 00/75278 A2

GENE ENCODING NADE, P75<sup>NTR</sup>-ASSOCIATED CELL DEATH EXECUTOR  
AND USES THEREOF

- 5 This application claims priority and is a continuation-in-part application of U.S. Serial No. 09/327,750, filed June 7, 1999, the contents of which is hereby incorporated by reference.
- 10 This invention described herein was supported by National Institutes of Health grant R01-GM55147. Accordingly, the United States Government has certain rights in this invention.
- 15 Throughout this application various publications are referred to within parenthesis. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications, in their entirety, are hereby incorporated by reference
- 20 into this application in order to more fully describe the state of the art to which this invention pertains.

Background of the Invention

- The low-affinity neurotrophin receptor (p75<sup>NTR</sup>) can mediate
- 25 cell survival or cell death by NGF or another neurotrophins stimulation in neuronal cells (1, 2, 3). To elucidate p75<sup>NTR</sup>-mediated signal transduction, the yeast two-hybrid system was employed to screen the mouse embryo cDNA libraries using the rat p75<sup>NTR</sup>ICD (intracellular domain) as a target. One
- 30 positive clone was identified and termed NADE (p75<sup>NTR</sup>-associated cell death executor). NADE has a significant homology to human HGR74 protein (4) and does not have a typical biochemical motif except the consensus sequences of nuclear export signal (NES)(5) and ubiquitination (6).
- 35 Expression of NADE mRNA was found highest in brain, heart, and lung. NADE specifically binds to p75<sup>NTR</sup>ICD both *in vitro* and *in vivo*. Co-expression of NADE together with p75<sup>NTR</sup> dramatically induced Caspase-2 and Caspase-3 activities to

cleave PARP (poly (ADP-ribose) polymerase) and fragmentation of nuclear DNA in 293T cells, but NADE without p75<sup>NTR</sup> did not show apoptosis, suggesting that NADE expression is necessary for p75<sup>NTR</sup>-mediated apoptosis but is not sufficient to trigger 5 apoptosis. Moreover, NGF dependent recruitment of NADE to p75<sup>NTR</sup>ICD was observed in a dose dependent manner and NADE significantly inhibits NF-kB activation. Interestingly, NADE protein is found to be ubiquitinated as a substrate for protein degradation pathway. Taken together, NADE is the 10 first signal adaptor molecule identified in involvement of p75<sup>NTR</sup>-mediated apoptosis, and it may play an important role in the pathogenesis of neurogenetic diseases.

**Summary of the Invention**

This invention provides an isolated nucleic molecule encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor.

5 This invention provides a method of producing a polypeptide capable of binding a p75<sup>NTR</sup> receptor which comprises growing host cells selected from a group consisting of bacterial, plant, insect or mammalian cell, under suitable conditions permitting production of the polypeptide.

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This invention provides an antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to an mRNA molecule encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor.

15

This invention provides a purified polypeptide capable of binding a p75<sup>NTR</sup> receptor.

This invention provides a method of producing a polypeptide  
20 capable of binding a p75<sup>NTR</sup> receptor into a suitable vector which comprises: (a) inserting a nucleic acid molecule encoding the polypeptide capable of binding a p75<sup>NTR</sup> receptor into a suitable vector; (b) introducing the resulting vector into a suitable host cell; (c) selecting the introduced host  
25 cell for the expression of the polypeptide capable of binding a p75<sup>NTR</sup> receptor; (d) culturing the selected cell to produce the polypeptide capable of binding a p75<sup>NTR</sup> receptor; and (e) recovering the polypeptide capable of binding a p75<sup>NTR</sup> receptor produced.

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This invention provides a method of identifying a compound capable of inhibiting binding between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> receptor, where said binding forms a complex between p75<sup>NTR</sup> receptor and a  
35 polypeptide capable of binding p75<sup>NTR</sup> receptor, comprising: a) contacting the compound under conditions permitting the binding of the polypeptide capable of binding p75<sup>NTR</sup> receptor



and p75<sup>NTR</sup> receptor with the polypeptide capable of binding p75<sup>NTR</sup> receptor to form a mixture; b) contacting p75<sup>NTR</sup> receptor with the mixture from step a); and c) measuring the amount of complexed p75<sup>NTR</sup> receptor and a polypeptide capable  
5 of binding p75<sup>NTR</sup> receptor.

This invention provides a method of identifying a compound capable of inhibiting binding between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> receptor, where said  
10 binding forms a complex between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> receptor, comprising:  
a) contacting the compound under conditions permitting the binding of the polypeptide capable of binding p75<sup>NTR</sup> receptor and p75<sup>NTR</sup> receptor with the p75<sup>NTR</sup> receptor to form a mixture;  
15 b) contacting the polypeptide capable of binding a p75<sup>NTR</sup> receptor with the mixture from step a); and c) measuring the amount of complexed p75<sup>NTR</sup> receptor and a polypeptide.

This invention provides a method of inducing apoptosis in  
20 cells which comprises expressing a polypeptide capable of binding a p75<sup>NTR</sup> receptor in the cells.

This invention provides a method of inducing apoptosis in a subject which comprises expressing a polypeptide capable of  
25 binding a p75<sup>NTR</sup> receptor in the subject.

This invention provides a method of determining physiological effects of expressing varying levels of a polypeptide capable of binding a p75<sup>NTR</sup> receptor in a transgenic nonhuman mammal  
30 which comprises producing a panel of transgenic nonhuman mammals, each nonhuman mammal expressing a different amount of polypeptide capable of binding a p75<sup>NTR</sup> receptor.

This invention provides a method of inducing apoptosis of  
35 cells in a subject comprising administering to the subject the purified polypeptide capable of binding a p75<sup>NTR</sup> receptor in an amount effective to induce apoptosis.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a subject with an appropriate amount of the compound; and (b) measuring the expression level of a polypeptide capable of binding a p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene in the subject, an increase of the expression levels of the polypeptide capable of binding a p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene indicating that the compound is an apoptosis inducing compound.

10 This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a cell with an appropriate amount of the compound; and (b) measuring the expression level of a polypeptide capable of binding a p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene in the cell, an increase of the  
15 expression levels of the polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup> gene indicating that the compound is an apoptosis inducing compound.

This invention provides a method for screening cDNA libraries  
20 of a polypeptide capable of binding a p75<sup>NTR</sup> receptor sequence using a yeast two-hybrid system and using a p75<sup>NTR</sup> intracellular domain as a target.

This invention provides a method to induce caspase-2 and  
25 caspase-3 activity to cleave poly (ADP-ribose) polymerase and fragment nuclear DNA in a cell by co-expression of a polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>.

This invention provides a method to inhibit NF- $\kappa$ B activation  
30 in a cell with a polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>.

This invention provides a method to detect a neurodegenerative disease in a subject by detecting  
35 expression levels of a polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>.

This invention provides a transgenic nonhuman mammal which

comprises an isolated nucleic acid, encoding a human HGR74 protein, which is a DNA molecule.

This invention provides a method of determining physiological effects of expressing varying levels of a human HGR74 protein in a transgenic nonhuman mammal which comprises producing a panel of transgenic nonhuman mammal, each nonhuman mammal expressing a different amount of human HGR74 protein.

10 This invention provides a method of producing the isolated human HGR74 protein into a suitable vector which comprises: (a) inserting a nucleic acid molecule encoding a human HGR74 protein into a suitable vector; (b) introducing the resulting vector into a suitable host cell; (c) selecting the  
15 introduced host cell for the expression of the human HGR74 protein; (d) culturing the selected cell to produce the human HGR74 protein; and (e) recovering the human HGR74 protein produced.

20 This invention provides a method of inducing apoptosis of cells in a subject comprising administering to the subject the purified human HGR74 protein in an amount effective to induce apoptosis.

25 This invention provides a pharmaceutical composition comprising a purified polypeptide capable of binding a p75<sup>NTR</sup> receptor and a pharmaceutically acceptable carrier.

This invention provides a method for identifying an apoptosis  
30 inducing compound comprising: (a) contacting a subject with an appropriate amount of the compound; and (b) measuring the expression level of human HGR74 protein gene and p75<sup>NTR</sup> gene in the subject, an increase of the expression levels of human HGR74 protein gene and p75<sup>NTR</sup> gene indicating that the  
35 compound is an apoptosis inducing compound.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a cell with an

appropriate amount of the compound; and (b) measuring the expression level of human HGR74 gene and p75<sup>NTR</sup> gene in the cell, an increase of the expression levels of human HGR74 protein gene and p75<sup>NTR</sup> gene indicating that the compound is  
5 an apoptosis inducing compound.

This invention provides a method for screening cDNA libraries human HGR74 sequence using a yeast two-hybrid system using a p75<sup>NTR</sup> intracellular domain as a target.

10

This invention provides a method to induce caspase-2 and caspase-3 activity to cleave poly (ADP-ribose) polymerase and fragment nuclear DNA in a cell by co-expression of human HGR74 protein and p75<sup>NTR</sup>.

15

This invention provides a method to inhibit NF- $\kappa$ B activation in a cell with human HGR74 protein and p75<sup>NTR</sup>.

This invention provides a method to detect a  
20 neurodegenerative disease in a subject by detecting expression levels of a polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>.

This invention provides a method of identifying a compound,  
25 which is an apoptosis inhibitor, said compound is capable of inhibiting specific binding between a polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup> receptor, so as to prevent apoptosis which comprises: (a) contacting the polypeptide capable of binding a p75<sup>NTR</sup> receptor with a plurality of  
30 compounds under conditions permitting binding between a known compound previously shown to be able to displace the polypeptide capable of binding a p75<sup>NTR</sup> receptor and the p75<sup>NTR</sup> receptor and the bound p75<sup>NTR</sup> receptor to form a complex; and  
(b) detecting the displaced polypeptide capable of binding a  
35 p75<sup>NTR</sup> receptor or the complex formed in step (a), wherein the displacement indicates that the compound is capable of inhibiting specific binding between the polypeptide capable of binding a p75<sup>NTR</sup> receptor and the p75<sup>NTR</sup> receptor.

This invention provides a method of identifying a compound, which is an apoptosis inhibitor, said compound is capable of inhibiting specific binding between human HGR74 protein and p75<sup>NTR</sup> receptor, so as to prevent apoptosis which comprises:  
5 (a) contacting the human HGR74 protein with a plurality of compounds under conditions permitting binding between a known compound previously shown to be able to displace the human HGR74 protein and the p75<sup>NTR</sup> receptor and the bound p75<sup>NTR</sup> receptor to form a complex; and (b) detecting the displaced  
10 human HGR74 protein or the complex formed in step (a), wherein the displacement indicates that the compound is capable of inhibiting specific binding between the human HGR74 protein and the p75<sup>NTR</sup> receptor.

15 This invention provides an isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the N-terminal 1-40 amino acids of wild type NADE polypeptide have  
20 been deleted and the deletion mutant is designated NADE N(41-124), and the NADE N(41-124) induces apoptosis in the presence of p75<sup>NTR</sup>.

This invention also provides an isolated nucleic acid  
25 molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the C-terminal 72-124 amino acids of wild type NADE polypeptide have been deleted and the deletion  
30 mutant is designated NADE N(1-71), and the NADE N(1-71) induces apoptosis in the presence of p75<sup>NTR</sup> and in the absence of p75<sup>NTR</sup>.

This invention further provides an isolated nucleic acid  
35 molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the N-terminal 1-40 amino acids and

the C-terminal 72-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(41-71), and the NADE N(41-71) induces apoptosis in the presence of p75<sup>NTR</sup> and in the absence of p75<sup>NTR</sup>.

This invention provides an isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the C-terminal 121-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(1-120) and the NADE N(1-120) induces apoptosis in the presence of p75<sup>NTR</sup>.

15

This invention also provides an isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the C-terminal 113-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(1-112) and the NADE N(1-112) induces apoptosis in the presence of p75<sup>NTR</sup>.

25 This invention further provides an isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the C-terminal 101-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(1-100) and the NADE N(1-100) induces apoptosis in the presence of p75<sup>NTR</sup> and in the absence of p75<sup>NTR</sup>.

35 This invention further provides an isolated nucleic acid molecule encoding a mutation of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated

10

neurotrophin associated cell death executor protein (NADE), wherein the point mutation results in Ala at amino acid position 99 for Leu at amino acid position of wild type NADE polypeptide, wherein the substitution mutant polypeptide is 5 designated NADE N(L99A) and the NADE N(L99A) induces apoptosis in the presence of p75<sup>NTR</sup>.

10

**Brief Description of Figures**

The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

5 C=cytosine  
A=adenosine  
T=thymidine  
G=guanosine

10 As used herein, amino acid residues are abbreviated as follows:

A=Alanine  
C=Cysteine  
15 D=Aspartic Acid  
E=Glutamic Acid  
F=Phenylalanine  
G=Glycine  
H=Histidine  
20 I=Isoleucine  
K=Lysine  
L=Leucine  
M=Methionine  
N=Asparagine  
25 P=Proline  
Q=Glutamine  
R=Arginine  
S=Serine  
T=Threonine  
30 V=Valine  
W=Tryptophan  
Y=Tyrosine



**Figure legends**

**Fig. 1 A-H** Amino acid sequence and expression analysis of NADE.

**Figure 1A**

5 Amino acid alignment of mouse and human NADE (HGR74) (4) proteins. The dotted sequence is asparagine rich stretch. The asterisks indicate the leucine-rich nuclear export signal (NES) (5). The closed triangle indicates cysteine residue essential for dimmer formation. The prenylation sequence in  
10 C-termini is underlined.

**Figure 1B**

Comparison of leucine-rich nuclear export signal (NES) (5) in various protein. The consensus sequence for NES are  
15 shadowed. Genbank accession numbers are: cZyxin, X69190; MAPKK, D13700; PKI-a, L02615; TFIIIA, M85211; RevHIV-1, AF075719; RanBP1, L25255; FMRP, L29074; Gle1, U68475; Human NADE, submitted; mouse NADE, submitted.

**20 Figure 1C**

Consensus sequence of ubiquitination signal.

**Figure 1D**

Northern blot analysis of NADE.

25

**Figure 1E**

Expression of endogenous NADE protein in SK-N-MC human neuroblastoma cells. SK-N-MC cell lysate treated with ALLN is immunoprecipitated by anti-NADE antibody, and subjected to  
30 immunoblotting by same antibody. Human NADE protein transiently expressed in 293T cells and untreated gels were used for controls. Heavy chain bands are resulted from antibodies using immunoprecipitation.

**35 Figure 1F**

Mutant analysis of mouse NADE protein A wild type NADE,

muNADE(Cys102Ser), and muNADE(Cys121Ser) proteins transiently expressed in 293T cells were detected by immunoblotting with anti-NADE antibody. Transfection methods are described in material and methods. The cell lysate extracted from the 5 293T cells transfected with parental vector was used as a control.

#### Figure 1G-1 and 1G-2

Blast Search and comparison of mouse NADE nucleic acid 10 sequence Figure 1G-1 (SEQ ID NO: \_\_) and human protein HGR74 sequence

#### Figure 1H

Comparison of mouse NADE, human HGR74 protein and other 15 homologous rat, mouse and human amino acid sequences

Fig. 2A-C NADE binds to p75<sup>NTR</sup> strongly *in vitro* and *in vivo*.

#### Figure 2A

20 *In vitro* binding assay of NADE and p75<sup>NTR</sup>. *In vitro*-translated NADE protein was subjected to GST-pull down assay using a GST-p75<sup>NTR</sup>ICD fusion protein. GST was used as a control.

#### 25 Figure 2B

*In vivo* binding assay of NADE and p75<sup>NTR</sup>. The cell lysates extracted from 293T cells co-transfected with Myc-tagged NADE and p75<sup>NTR</sup> were co-immunoprecipitated by anti-Myc antibody, and subjected to immunoblotting by anti-p75<sup>NTR</sup> antibody. The 30 lysates from the cells transfected with each plasmid and a parental vector were used as controls. Transfection methods are described in material and methods.

#### Figure 2C

35 Interaction of NADE with p75<sup>NTR</sup> depending on NGF ligation. 293T cells co-transfected with Myc-tagged NADE and p75<sup>NTR</sup> were

treated with NGF in various concentration as indicated. Upper panel; Immunoprecipitates of anti-Myc antibody (IgG1) from each sample were subjected to immunoblotting analysis by anti p75<sup>NTR</sup> antibody. Middle and lower panels indicated the expression level of p75<sup>NTR</sup> and NADE proteins by immunoblotting, respectively. The immunoprecipitate of anti-FLAG antibody (IgG1) was used as a control.

**Fig. 3A-E** Effect of NADE and p75<sup>NTR</sup> co-transfection on 293T cells.

**Figure 3A**

Morphological change caused by co-transfection of NADE and p75<sup>NTR</sup> in 293T cells transfected by each cDNA were observed 48 hours after transfection. The magnification was 200. Transfection methods are described in material and methods.

**Figure 3B**

TUNEL assay. Transfected 293T cells were stained by TUNEL method and analyzed by a flow cytometer. The percentages indicated are positive populations.

**Figure 3C**

DNA fragmentation analysis. DNAs from transfected 293T cells were checked by 1.5 % agarose gel electrophoresis.

**Figure 3D**

Inhibition of NF- $\kappa$ B activity by NADE. NF- $\kappa$ B activities in transfected cells were measured by E-selectin promoter-luciferase gene reporter assay. Luciferase activities were determined 24 hours after transfection and normalized on the basis of pRL-TK expression levels.

**Figure 3E**

Activation of Caspase-2 and 3 and degradation of PARP in co-transfected 293T cells. The cell extracts from 293T cells transfected by each cDNA as indicated were analyzed by

immunoblotting with anti-Caspase-2, Caspase-3, and PARP antibody. The level of  $\alpha$ -tubulin was measured as a control.

**Fig. 4A-D** A conserved Rev-like NES in the C-terminus mediates nuclear export of NADE protein.

**Figure 4A**

At residues 88-100, the mouse NADE NES lies within the C-terminus. A mouse NADE is aligned with homologous sequences of NADE family members and the NES sequences of HIV Rev, MAPKK, cZyxin and PKI-a.

**Figure 4B**

Subcellular localization of a wild type mNADE-GFP and a control GFP vector was analyzed in transfected 293T cells.

**Figure 4C**

Effects of deletion mutants of NES motif on nuclear export of GFP-fused mouse NADE proteins. Both deletion mutants with or without NES indicate deletion-124 and delta 91-124, respectively.

**Figure 4D**

Effects of point mutations within the NES motif on nuclear export of GFP-fused mouse NADE proteins. The single or double amino acid substitutions were made at residue 94 and 97 (Leu to Ala). GFP-constructs were transiently transfected into 293T cells. The fixed cells were stained with TO-PRO-3 to visualize the nucleus and images of representative cell fields were captured on a confocal laser microscope. More than 1000 cells were analyzed for each construct.

-16-

**FIGURE LEGENDS**

**Fig. 5 Schematic representation of NADE mutants.** A, Deletion mutants of NADE. The domain structure of full length mouse NADE is shown at the top. Amino acid numbers are listed above: Nuclear export signal (*NES*) (90-100) and Ubiquitin sequence (*US*) (91-112) domains. The various NADE deletion mutants are indicated diagrammatically. B, Point mutants of mNADE. Mutations in the C-terminus and *NES*. Schematic representation of C-terminal half domain sequences in wild-type NADE and mutants.

**Fig. 6 NGF-dependent regulation of p75NTR/NADE-induced apoptosis in 293T cells.** A, Morphological analysis in 293T cells. Cells were transiently transfected with pcDNA3/rat-p75NTR or/and pcDNA3.1/myc-His(-)A/mNADE (WT) and cultured for 10 h. After withdrawing the serum, cells were treated with or without 100 ng/ml NGF for 36 h. Cells were fixed with 3.7 % paraformaldehyde and nuclear morphology was analyzed by DAPI staining. B, Percentage of apoptotic cells determined by DAPI staining. Number of apoptotic cells with nuclear morphology typical of apoptosis was scored in at least 400 cells in each sample by using a fluorescence microscope. The data shown are the percentage of apoptotic cells (mean  $\pm$  S. D.) from individual four experiments.

**Fig. 7 Mapping analysis of NADE for apoptosis.** Mutational analysis of NADE for apoptosis. The indicated construct were transiently transfected with or without p75NTR into 293T cells. Cells were fixed with 3.7 % paraformaldehyde and nuclear morphology was analyzed by DAPI staining. Data (mean  $\pm$  S. D.) shown are the percentage of apoptotic cells among the total number of cells counted (n = 4).

-17-

**Fig. 8 Effect of NADE NES function.** *A*, A conserved Rev-like NES in NADE. NADE is aligned with homologous sequences of NADEs and the NESs of PKI, HIV, Rev, MDM2 and MAPKK. *B*, Subcellular localization analysis of NES mutants in 293T cells. Cells were transfected with GFP-vector, GFP-NADE (WT), GFP-N (L99A) and GFP-N (L94A, L97A, L99A). To-PRO-3 iodide was used to visualize the nucleus, and the subcellular localization analysis was performed as described under "Experimental Procedures." *C*, Dimer formation of NADE. 293T cells were transfected with pcDNA3.1/myc-His(-)A/mNADE (WT), pcDNA3.1/myc-His(-)A/N (L99A) and pcDNA3.1/myc-His(-)A/N (L94A, L97A, L99A) for 36 h. Cells were lysed in lysis buffer and centrifuged at 16,000 g for 30 min. The resultant supernatant was boiled in SDS-PAGE sample buffer without or with 50  $\mu$ M 2-mercaptoethanol (2-ME) for 5 min, subjected to a 12.5 % SDS-PAGE and analyzed by Western blot with the anti-NADE polyclonal antibody. *D*, Interaction of NADE and its point mutants with p75NTR. The interaction of mutants with p75NTR was measured by using the GST fusion protein containing the p75NTR cytoplasmic region (338-396) with either NADE or its NES mutants; the NADE constructs had been translated in vitro and labeled with [<sup>35</sup>S] methionine. Bound complexes were precipitated as described in Materials and Methods. *E*, Effect of mutation in NES on apoptosis. The indicated constructs were transiently transfected with p75NTR into 293T cells. Cells were fixed with 3.7 % paraformaldehyde and nuclear morphology was analyzed by DAPI staining. Data (mean  $\pm$  S. D.) shown are the percentage of apoptotic cells among the total number of cells counted (n = 4).

**Fig. 9 NADE suppresses NF- $\kappa$ B activity.** *A*, Overexpression of NADE suppresses basal NF- $\kappa$ B activity in 293T cells. 293T cells were transiently cotransfected with an E-

selectin-luciferase reporter gene, a pRL-TK, pCDNA3/p75NTR and pCDNA3.1myc-His/mNADE WT (0.3, 3.0  $\mu$ g). 12 h post-transfection, cells were either left untreated or treated with 100 ng/ml NGF for 24 h. Double Luciferase reporter system were used to normalize luciferase values for transfection efficiency. B, PC12 cells were transfected with the E-selectin-luciferase reporter plasmid, pR-TK and the wild-type NADE expression plasmid (0.3, 1.0  $\mu$ g) or vector alone and cultured for 24 h in the presence of 100 ng/ml NGF. Values shown represent luciferase activities relative to vector control and shown as the means (bars, S.D.) of experiments performed in triplicate. C, nmr5 cells were transfected with the E-selectin-luciferase reporter plasmid, pR-TK and the wild-type mNADE expression plasmid (0.3, 1.0  $\mu$ g) or vector alone and cultured for 24 h in the presence of 100 ng/ml NGF. Values shown represent luciferase activities relative to vector control and shown as the means (bars, S.D.) of experiments performed in triplicate.

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**Fig. 10 Dominant negative effect of NADE mutants on NF- $\kappa$ B activity in 293T cells.** Cells were transfected with the E-selectin-luciferase reporter plasmid, pR-TK and the indicated NADE mutant expression plasmid (0.3, 1.0, 3.0  $\mu$ g) or vector alone and incubated for 24 h. Values shown represent luciferase activities relative to vector control and shown as the means (bars, S.D.) of experiments performed in triplicate.

**Fig. 11 Schematic representation of functional domains of NADE.** The domain structure of full length mouse NADE is shown at the top. Amino acid numbers are listed above: Nuclear export signal (NES) (90-100) and Ubiquitin sequence (US) (91-112) domains. Pro-apoptotic domain resides between 41 and 71. Regulatory domain resides

35

-19-

between 72 and 112, containing p75NTR-binding domain (81-106). Two domains (61-90, 121-124) contribute to NF- B suppression.



**Detailed Description of the Invention**

The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

5

C=cytosine

A=adenosine

T=thymidine

G=guanosine

10

As used herein, amino acid residues are abbreviated as follows:

A=Alanine

15 C=Cysteine

D=Aspartic Acid

E=Glutamic Acid

F=Phenylalanine

G=Glycine

20 H=Histidine

I=Isoleucine

K=Lysine

L=Leucine

M=Methionine

25 N=Asparagine

P=Proline

Q=Glutamine

R=Arginine

S=Serine

30 T=Threonine

V=Valine

W=Tryptophan

Y=Tyrosine

35 This invention provides an isolated nucleic molecule encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor. In an embodiment of the above described isolated nucleic molecule

encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor the isolated nucleic acid is a DNA molecule. In another embodiment of the above described isolated nucleic acid molecule encoding a polypeptide capable of binding a p75<sup>NTR</sup> 5 receptor the isolated nucleic acid is a cDNA molecule. In a further embodiment of the above described isolated DNA molecule encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor the isolated nucleic acid is a RNA molecule. In an embodiment of the above described isolated nucleic acid 10 molecule encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor, the isolated nucleic acid is operatively linked to a promoter of RNA transcription. In yet another embodiment of the above described nucleic acid molecule, said isolated nucleic acid molecule encodes a neurotrophin associated cell 15 death executor protein. In an embodiment of the above described nucleic acid molecule, said isolated nucleic acid molecule comprises a sequence of AATTG TCTAC GCATC CTTAT GGGGG AGCTG TCTAA C.

20 As used herein, "polypeptide" includes both peptides and proteins. "Peptide" means a polypeptide of fewer than 10 amino acid residues in length, and "protein" means a polypeptide of 10 or more amino acid residues in length. In this invention, the polypeptides may be naturally occurring 25 or recombinant (i.e. produced via recombinant DNA technology), and may contain mutations (e.g. point, insertion and deletion mutations) as well as other covalent modifications (e.g. glycosylation and labeling [via biotin, streptavidin, fluoracine, and radioisotopes such as <sup>131</sup>I]). 30 Moreover, each instant composition may contain more than a single polypeptide, i.e., each may be a monomer (one polypeptide bound to a polymer) or a multimer (two or more polypeptides bound to a polymer or to each other).

35 The p75<sup>NTR</sup> receptor is a low affinity nerve growth factor (NGF) receptor with a low affinity to neurotrophins. p75<sup>NTR</sup>

receptor has been implicated in the mediation of cell death and cell survival.

"Capable of binding" is defined as the ability of a protein or other peptide molecule capable of recognizing and interacting with a complementary receptor site, which can be another protein or other type of molecule.

The DNA molecules of the subject invention also include DNA molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide capable of binding a p75<sup>NTR</sup> receptor, and as products for the large scale synthesis of the polypeptide capable of binding a p75<sup>NTR</sup> receptor, or fragments thereof, by a variety of recombinant techniques. The DNA molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of

the polypeptide capable of binding a p75<sup>NTR</sup> receptor or portions thereof and related products.

This invention provides a vector which comprises the isolated  
5 nucleic acid encoding a polypeptide capable of binding a  
p75<sup>NTR</sup> receptor, operatively linked to a promoter of RNA  
transcription. In an embodiment of the invention, where in  
the vector which comprises the isolated nucleic acid encoding  
a polypeptide capable of binding a p75<sup>NTR</sup> receptor,  
10 operatively linked to a promoter of RNA transcription is a  
plasmid. In another embodiment the above described isolated  
nucleic acid molecule which is a cDNA molecule encoding a  
polypeptide capable of binding a p75<sup>NTR</sup> receptor, encodes a  
human or mouse protein. In yet another embodiment the above  
15 described isolated nucleic acid molecule is a cDNA molecule  
wherein the nucleic acid molecule encodes a polypeptide  
capable of binding a p75<sup>NTR</sup> receptor comprising the amino acid  
sequence set forth in Figure 1G-1 (SEQ ID NO: \_\_\_\_). In a  
further embodiment the above described isolated nucleic acid  
20 molecule is a cDNA molecule wherein the nucleic acid molecule  
encodes a polypeptide capable of binding a p75<sup>NTR</sup> receptor.  
In an embodiment of the above described isolated nucleic acid  
molecule which is a cDNA molecule wherein the nucleic acid  
molecule encodes a polypeptide capable of binding a p75<sup>NTR</sup>  
25 receptor which is a mouse, rat or human protein. In yet  
another embodiment of the above described isolated nucleic  
acid molecule which is a cDNA molecule, said isolated nucleic  
acid molecule comprises the nucleic acid sequence set forth  
in Figure 1G-1 (SEQ ID NO: \_\_\_\_).

30

Numerous vectors for expressing the inventive proteins may be  
employed. Such vectors, including plasmid vectors, cosmid  
vectors, bacteriophage vectors and other viruses, are well  
known in the art. For example, one class of vectors utilizes  
35 DNA elements which are derived from animal viruses such as  
bovine papilloma virus, polyoma virus, adenovirus, vaccinia

virus, baculovirus, retroviruses (RSV, MMTV or MoMLV), Semliki Forest virus or SV40 virus. Additionally, cells which have stably integrated the DNA into their chromosomes may be selected by introducing one or more markers which  
5 allow for the selection of transfected host cells. The markers may provide, for example, prototrophy to an auxotrophic host, biocide resistance or resistance to heavy metals such as copper. The selectable marker gene can be either directly linked to the DNA sequences to be expressed,  
10 or introduced into the same cell by cotransformation.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. Additional elements may also  
15 be needed for optimal synthesis of mRNA. These additional elements may include splice signals, as well as enhancers and termination signals. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the  
20 start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or  
25 assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general.

These vectors may be introduced into a suitable host cell to  
30 form a host vector system for producing the inventive proteins. Methods of making host vector systems are well known to those skilled in the art.

Methods of introducing nucleic acid molecules into cells are  
35 well known to those of skill in the art. Such methods include, for example, the use of viral vectors and calcium

phosphate co-precipitation.

This invention provides a host cell comprising the vector comprising the nucleic acid molecule of encoding a polypeptide capable of binding p75<sup>NTR</sup> receptor. In an embodiment the above described host cell is selected from a group consisting of a bacterial cell, a plant cell, and insect cell, and a mammalian cell.

10 The "suitable host cell" in which the nucleic acid molecule encoding is a polypeptide capable of binding a p75<sup>NTR</sup> receptor capable of being expressed is any cell capable of taking up the nucleic acid molecule and stably expressing the polypeptide capable of binding a p75<sup>NTR</sup> receptor encoded  
15 thereby.

Suitable host cells include, but are not limited to, bacterial cells (including gram positive cells), yeast cells, fungal cells, insect cells and animal cells. Suitable animal  
20 cells include, but are not limited to HeLa cells, C<sub>6</sub> cells, CV1 cells and various primary mammalian cells. Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH-3T3 cells, CHO cells, HeLa cells, Ltk<sup>-</sup> cells and COS cells. Mammalian cells  
25 may be transfected by methods well known in the art such as calcium phosphate precipitation, electroporation and microinjection.

This invention provides a method of producing a polypeptide  
30 having the biological activity of a polypeptide capable of binding a p75<sup>NTR</sup> receptor which comprises growing host cells selected from a group consisting of bacterial, plant, insect or mammalian cell, under suitable conditions permitting production of the polypeptide. In another embodiment of the  
35 above described method of producing a polypeptide having the biological activity of a polypeptide capable of binding a

p75<sup>NTR</sup> receptor, the method further comprises the recovering of the produced polypeptide.

This invention provides an isolated nucleic acid molecule of  
5 at least 15 contiguous nucleotides capable of specifically  
hybridizing with a unique sequence included within the  
sequence of the nucleic acid molecule encoding a polypeptide  
capable of binding a p75<sup>NTR</sup> receptor. In an embodiment of the  
above described isolated nucleic acid molecule of at least 15  
10 contiguous nucleotides capable of specifically hybridizing  
with a unique sequence included within the sequence of the  
nucleic acid molecule encoding a polypeptide capable of  
binding a p75<sup>NTR</sup> receptor, said isolated nucleic acid molecule  
is a DNA molecule. In another embodiment of the above  
15 described isolated nucleic acid molecule of at least 15  
contiguous nucleotides capable of specifically hybridizing  
with a unique sequence included within the sequence of the  
nucleic acid molecule encoding a polypeptide capable of  
binding a p75<sup>NTR</sup> receptor, said isolated nucleic molecule is  
20 a RNA molecule.

This invention provides an isolated nucleic acid molecule  
capable of specifically hybridizing with a unique sequence  
included within the sequence of a nucleic acid molecule which  
25 is complementary to the nucleic acid molecule encoding a  
polypeptide capable of binding a p75<sup>NTR</sup> receptor. In an  
embodiment the above described isolated nucleic acid molecule  
which is complementary to the nucleic acid molecule encoding  
a polypeptide capable of binding a p75<sup>NTR</sup> receptor is a DNA  
30 molecule. In another embodiment the above described isolated  
nucleic acid molecule capable of specifically hybridizing  
with a nucleic acid molecule capable of specifically  
hybridizing with a unique sequence included within the  
sequence of a nucleic acid molecule which is complementary to  
35 the nucleic acid molecule encoding a polypeptide capable of  
binding a p75<sup>NTR</sup> receptor is a RNA molecule.

One of ordinary skill in the art will easily obtain unique sequences from the cDNA cloned in the polypeptide capable of binding a p75<sup>NTR</sup> receptor plasmid. Such unique sequences may be used as probes to screen various mammalian cDNA libraries 5 and genomic DNAs, e.g. mouse, rat and bovine, to obtain homologous nucleic acid sequences and to screen different cDNA tissue libraries to obtain isoforms of the obtained nucleic acid sequences. Nucleic acid probes from the cDNA cloned in the polypeptide capable of binding a p75<sup>NTR</sup> receptor 10 plasmid may further be used to screen other human tissue cDNA libraries to obtain isoforms of the nucleic acid sequences encoding polypeptide capable of binding a p75<sup>NTR</sup> receptor as well as to screen human genomic DNA to obtain the analogous nucleic acid sequences. The homologous nucleic acid sequences 15 and isoforms may be used to produce the proteins encoded thereby.

As used herein, "capable of specifically hybridizing" means capable of binding to an mRNA molecule encoding a polypeptide 20 capable of binding a p75<sup>NTR</sup> receptor but not capable of binding to a polypeptide capable of binding a p75<sup>NTR</sup> receptor molecule encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor.

25 This invention provides an antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to an mRNA molecule encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor. In an embodiment of the above described antisense oligonucleotide, said antisense oligonucleotide has 30 a nucleic acid sequence capable of specifically hybridizing to the isolated cDNA molecule encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor. In another embodiment of the above described antisense oligonucleotide has a nucleic acid sequence capable of specifically hybridizing to the isolated 35 RNA molecule encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor.



This invention provides a purified a polypeptide capable of binding a p75<sup>NTR</sup> receptor. In an embodiment of the above described purified polypeptide capable of binding p75<sup>NTR</sup> receptor is encoded by the isolated nucleic acid encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor. In an embodiment the above described polypeptide capable of binding a p75<sup>NTR</sup> receptor is a fragment of the purified polypeptide capable of binding a p75<sup>NTR</sup> receptor. In another embodiment the above described purified polypeptide capable of binding a p75<sup>NTR</sup> receptor has substantially the same amino acid sequence as set forth in Figure 1G-1 (SEQ ID NO: \_\_\_\_). In a further embodiment the above described purified polypeptide capable of binding a p75<sup>NTR</sup> receptor having an amino acid sequence as set forth in Figure 1G-1 (SEQ ID NO: \_\_\_\_). In yet another embodiment the above described polypeptide capable of binding a p75<sup>NTR</sup> receptor has an amino acid sequence as set forth in Figure 1G-1 (SEQ ID NO: \_\_\_\_). In a further embodiment, the above described polypeptide capable of binding a p75<sup>NTR</sup> receptor is a vertebrate polypeptide capable of binding a p75<sup>NTR</sup> receptor. In an embodiment of the above described polypeptide capable of binding a p75<sup>NTR</sup> receptor comprises a neurotrophin associated cell death executor protein. In yet another embodiment of the above described polypeptide capable of binding a p75<sup>NTR</sup> receptor comprises NCLRILMGELSN.

As used herein, purified polypeptides means the polypeptides free of any other polypeptides.

As used herein, a polypeptide capable of binding a p75<sup>NTR</sup> receptor having "substantially the same" amino acid sequences as set forth in Figure 1G-1 (SEQ ID NO: \_\_\_\_ ) is encoded by a nucleic acid encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor, said nucleic acid having 100% identity in the homeodomain regions, that is those regions coding the protein, and said nucleic acid may vary in the nucleotides in

the non-coding regions.

This invention provides a monoclonal antibody directed to an epitope of a polypeptide capable of binding a p75<sup>NTR</sup> receptor.

5 In an embodiment the above described monoclonal antibody, said monoclonal antibody is directed to a mouse, rat or human polypeptide capable of binding a p75<sup>NTR</sup> receptor.

The term "antibody" includes, by way of example, both  
10 naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies, wholly synthetic antibodies, and fragments thereof. Optionally, an  
15 antibody can be labeled with a detectable marker. Detectable markers include, for example, radioactive or fluorescent markers.

This invention provides a polyclonal antibody directed to an  
20 epitope of the purified protein having the amino sequence as set forth in Figure 1G-1 (SEQ ID NO: \_\_\_\_). In a further embodiment the above described monoclonal or polyclonal antibodies are directed to the polypeptide capable of binding a p75<sup>NTR</sup> receptor, having the amino sequence as set forth in  
25 Figure 1G-1 (SEQ ID NO: \_\_\_\_).

Polyclonal antibodies may be produced by injecting a host animal such as rabbit, rat, goat, mouse or other animal with the immunogen of this invention, e.g. a purified mammalian  
30 polypeptide capable of binding a p75<sup>NTR</sup> receptor or a purified human polypeptide capable of binding a p75<sup>NTR</sup> receptor. The sera are extracted from the host animal and are screened to obtain polyclonal antibodies which are specific to the immunogen. Methods of screening for polyclonal antibodies  
35 are well known to those of ordinary skill in the art such as those disclosed in Harlow & Lane, Antibodies: A Laboratory

Manual, (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY: 1988) the contents of which are hereby incorporated by reference.

5 The monoclonal antibodies may be produced by immunizing for example, mice with an immunogen. The mice are inoculated intra-peritoneally with an immunogenic amount of the above-described immunogen and then boosted with similar amounts of the immunogen. Spleens are collected from the immunized mice  
10 a few days after the final boost and a cell suspension is prepared from the spleens for use in the fusion.

In the practice of the subject invention any of the above-described antibodies may be labeled with a detectable marker.  
15 In one embodiment, the labeled antibody is a purified labeled antibody. As used in the subject invention, the term "antibody" includes, but is not limited to, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and  
20 monoclonal antibodies, and binding fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof.

Furthermore, the term "antibody" includes chimeric antibodies  
25 and wholly synthetic antibodies, and fragments thereof. A "detectable moiety" which functions as detectable labels are well known to those of ordinary skill in the art and include, but are not limited to, a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or  
30 a label which may be detected through a secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase, horseradish peroxidase,  $\beta$ -galactosidase, fluorescein or streptavidin/biotin. Methods of labeling antibodies are well  
35 known in the art.

Determining whether the antibody forms such a complex may be accomplished according to methods well known to those skilled in the art. In the preferred embodiment, the determining is accomplished according to flow cytometry methods.

5

The antibody may be bound to an insoluble matrix such as that used in affinity chromatography. As used in the subject invention, isolating the cells which form a complex with the immobilized monoclonal antibody may be achieved by standard  
10 methods well known to those skilled in the art. For example, isolating may comprise affinity chromatography using immobilized antibody.

Alternatively, the antibody may be a free antibody. In this  
15 case, isolating may comprise cell sorting using free, labeled primary or secondary antibodies. Such cell sorting methods are standard and are well known to those skilled in the art.

20 The labeled antibody may be a polyclonal or monoclonal antibody. In one embodiment, the labeled antibody is a purified labeled antibody. The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody"  
25 includes polyclonal and monoclonal antibodies, and fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. The detectable marker may be, for example, radioactive or fluorescent. Methods of labeling antibodies  
30 are well known in the art.

This invention provides a method of inducing apoptosis in cells which comprises expressing polypeptide capable of binding a p75<sup>NTR</sup> receptor in the cells.

35

This invention provides a method of inducing apoptosis in a

subject which comprises expressing a polypeptide capable of binding a p75<sup>NTR</sup> receptor in the subject. In a further embodiment of the method of inducing apoptosis in a subject where the subject is a rat, mouse or human.

5

As used herein, "subject" means any animal or artificially modified animal. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. In the preferred embodiment, the subject is a human.

10

This invention provides a transgenic nonhuman mammal which comprises an isolated nucleic acid, encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor, which is a DNA molecule. In an embodiment of the above described transgenic nonhuman mammal, the DNA encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor is operatively linked to tissue specific regulatory elements.

This invention provides a method of determining physiological effects of expressing varying levels of a polypeptide capable of binding a p75<sup>NTR</sup> receptor in a transgenic nonhuman mammal which comprises producing a panel of transgenic nonhuman mammals, each nonhuman mammal expressing a different amount of a polypeptide capable of binding a p75<sup>NTR</sup> receptor.

25

This invention provides a method of producing a polypeptide capable of binding a p75<sup>NTR</sup> receptor into a suitable vector which comprises: (a) inserting a nucleic acid molecule encoding the polypeptide capable of binding a p75<sup>NTR</sup> receptor into a suitable vector; (b) introducing the resulting vector into a suitable host cell; (c) selecting the introduced host cell for the expression of the polypeptide capable of binding a p75<sup>NTR</sup> receptor; (d) culturing the selected cell to produce the polypeptide capable of binding a p75<sup>NTR</sup> receptor; and (e) recovering the polypeptide capable of binding a p75<sup>NTR</sup> receptor produced.

This invention provides a method of inducing apoptosis of cells in a subject comprising administering to the subject the purified polypeptide capable of binding a p75<sup>NTR</sup> receptor in an amount effective to induce apoptosis. In an embodiment 5 of the above described method of inducing apoptosis of cells in a subject comprising administering to the subject the purified polypeptide capable of binding a p75<sup>NTR</sup> receptor in an amount effective to induce apoptosis, the subject is a mammal. In another embodiment of the above-described method 10 of inducing apoptosis of cells in a subject, the subject is a mouse, rat or human.

As used herein "apoptosis" means programmed cell death of the cell. The mechanisms and effects of programmed cell death 15 differs from cell lysis. Some observable effects of apoptosis are: DNA fragmentation and disintegration into small membrane-bound fragments called apoptotic bodies.

As used herein, "subject" means any animal or artificially 20 modified animal. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. In the preferred embodiment, the subject is a human.

This invention provides a pharmaceutical composition 25 comprising a purified polypeptide capable of binding a p75<sup>NTR</sup> receptor and a pharmaceutically acceptable carrier.

The invention also provides a pharmaceutical composition comprising a effective amount of the polypeptides capable of 30 binding a p75<sup>NTR</sup> receptor described above and a pharmaceutically acceptable carrier. In the subject invention an "effective amount" is any amount of above-described polypeptides capable of binding a p75<sup>NTR</sup> receptor which, when administered to a subject suffering from a 35 disease or abnormality against which the proteins are determined to be potentially therapeutic, are effective,

causes reduction, remission, or regression of the disease or abnormality. In the practice of this invention the "pharmaceutically acceptable carrier" is any physiological carrier known to those of ordinary skill in the art useful in  
5 formulating pharmaceutical compositions.

In one preferred embodiment the pharmaceutical carrier may be a liquid and the pharmaceutical composition would be in the form of a solution. In another equally preferred embodiment,  
10 the pharmaceutically acceptable carrier is a solid and the composition is in the form of a powder or tablet. In a further embodiment, the pharmaceutical carrier is a gel and the composition is in the form of a suppository or cream. In a further embodiment the compound may be formulated as a part  
15 of a pharmaceutically acceptable transdermal patch.

A solid carrier can include one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids,  
20 binders or tablet-disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression  
25 properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active ingredient. Suitable solid carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose,  
30 polyvinylpyrrolidone, low melting waxes and ion exchange resins.

Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The  
35 active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an

organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration. The liquid carrier for pressurized compositions can be halogenated hydrocarbon or other pharmaceutically acceptable propellant.

20

Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized by for example, intramuscular, intrathecal, epidural, intraperitoneal or subcutaneous injection. Sterile solutions can also be administered intravenously. The compounds may be prepared as a sterile solid composition which may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium. Carriers are intended to include necessary and inert binders, suspending agents, lubricants, flavorants, sweeteners, preservatives, dyes, and coatings.

The above described pharmaceutical composition comprising a polypeptide capable of binding a p75<sup>NTR</sup> receptor can be administered orally in the form of a sterile solution or suspension containing other solutes or suspending agents, for



example, enough saline or glucose to make the solution isotonic, bile salts, acacia, gelatin, sorbitan monoleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like.

5

The above described pharmaceutical composition comprising a polypeptide capable of binding a p75<sup>NTR</sup> receptor can also be administered orally either in liquid or solid composition form. Compositions suitable for oral administration include  
10 solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

15

Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the particular above described pharmaceutical composition comprising a polypeptide capable of binding a p75<sup>NTR</sup> receptor in use, the strength of  
20 the preparation, the mode of administration, and the advancement of the disease condition or abnormality. Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of  
25 administration.

As used herein, administering may be effected or performed using any of the various methods known to those skilled in the art. The administration may be intravenous,  
30 intraperitoneal, intrathecal, intralymphatic, intramuscular, intralesional, parenteral, epidural, subcutaneous; by infusion, liposome-mediated delivery, aerosol delivery; topical, oral, nasal, anal, ocular or otic delivery.

35

A method of identifying a compound capable of inhibiting binding between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> receptor comprising: a) contacting the compound with the polypeptide capable of binding to p75<sup>NTR</sup> receptor under conditions permitting the binding of the polypeptide capable of binding to p75<sup>NTR</sup> receptor and p75<sup>NTR</sup> receptor to form a complex; b) contacting the p75<sup>NTR</sup> receptor with the mixture from step a); and c) measuring the amount of the formed complexes or the unbound p75<sup>NTR</sup> receptor or the unbound polypeptide or any combination thereof. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a neurotrophin associated cell death executor. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a human HGR74 protein. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a musnade3a sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a hunade3a1 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a hunade3a2 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a ratnad3a sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable

of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a ratnad3b sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a musnade3b sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a humnadel sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a ratnadel sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a musnadel sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a humnade2 sequence as defined on Figure 1H.

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A method of identifying a compound capable of inhibiting binding between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> receptor, where said binding forms a complex between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> receptor, comprising: a) contacting the compound with the p75<sup>NTR</sup> receptor under conditions permitting the binding of the polypeptide capable of binding to p75<sup>NTR</sup> receptor and p75<sup>NTR</sup> receptor to form a complex; b) contacting the p75<sup>NTR</sup> receptor with the mixture from step a); and c) measuring the amount of the formed complexes or the unbound p75<sup>NTR</sup> receptor or the unbound polypeptide or any combination thereof.

In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and

a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a neurotrophin associated cell death executor protein. In an embodiment of the above described method of identifying a compound capable of  
5 inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a human HGR74 protein. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable  
10 of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a musnade3a sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide  
15 capable of binding p75<sup>NTR</sup> is a hunade3a1 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> a hunade3a2  
20 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> a ratnad3a sequence as defined on Figure 1H. In an  
25 embodiment of the above described method of identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a ratnad3b sequence as defined on Figure 1H. In an embodiment of the above described method of  
30 identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a musnade3b sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of  
35 inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a humnade1 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a

compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a ratnadel sequence as defined on Figure 1H. In an embodiment of the above described method of  
5 identifying a compound capable of inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a musnadel sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of  
10 inhibiting between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> where said polypeptide capable of binding p75<sup>NTR</sup> is a humnade2 sequence as defined on Figure 1H.

This invention provides a method for identifying an apoptosis  
15 inducing compound comprising: (a) contacting a subject with an appropriate amount of the compound; and (b) measuring the expression level of polypeptide capable of binding a p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene in the subject, an increase of the expression levels of a polypeptide capable of binding a  
20 p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene indicating that the compound is an apoptosis inducing compound. In an embodiment of the above described method for identifying an apoptosis inducing compound comprising: a) contacting a subject with an appropriate amount of the compound; and (b) measuring the  
25 expression level of polypeptide capable of binding a p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene in the subject, an increase of the expression levels of a polypeptide capable of binding a p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene indicating that the compound is an apoptosis inducing compound, wherein the  
30 subject is a mammal. In an embodiment of the above-described method of identifying an apoptosis inducing compound, wherein the mammal subject is a mouse, rat or human.

This invention provides a method for identifying an apoptosis  
35 inducing compound comprising: (a) contacting a cell with an appropriate amount of the compound; and (b) measuring the

expression level of polypeptide capable of binding a p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene in the cell, an increase of the expression levels of polypeptide capable of binding a p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene indicating that the compound is  
5 an apoptosis inducing compound.

An apoptosis inducing compound is defined as a compound which may be, but not limited to, antibodies, inorganic compounds, organic compounds, peptides, peptidomimetic compounds,  
10 polypeptides or proteins, fragments or derivatives which share some or all properties, e.g. fusion proteins, that induces apoptosis. The compounds may be naturally occurring and obtained by purification, or may be non-naturally occurring and obtained by synthesis.

15

This invention provides a method for screening cDNA libraries encoding a polypeptide capable of binding a p75<sup>NTR</sup> receptor sequence using a yeast two-hybrid system and using a p75<sup>NTR</sup> intracellular domain as a target. In an embodiment of the  
20 above described method for screening cDNA libraries for polypeptide capable of binding a p75<sup>NTR</sup> receptor sequence using a yeast two-hybrid system and using a p75<sup>NTR</sup> intracellular domain as a target, where the cDNA library is mammalian. In another embodiment of the above described  
25 method for screening cDNA libraries for a polypeptide capable of binding a p75<sup>NTR</sup> receptor using a yeast two-hybrid system and using a p75<sup>NTR</sup> intracellular domain as a target, where the cDNA library is mammalian and where the mammalian cDNA library is derived from rat, mouse or human cDNA libraries.  
30 In an embodiment of the above described method for screening cDNA libraries for a polypeptide capable of binding a p75<sup>NTR</sup> receptor, using a yeast two-hybrid system and using a p75<sup>NTR</sup> intracellular domain as a target, where the p75<sup>NTR</sup> intracellular domain target is mammalian. In an embodiment  
35 of the above described method for screening cDNA libraries for a polypeptide capable of binding a p75<sup>NTR</sup> receptor using

a yeast two-hybrid system and using a p75<sup>NTR</sup> intracellular domain as a target, where the p75<sup>NTR</sup> intracellular domain target is a rat, mouse or human p75<sup>NTR</sup> intracellular domain target.

5

This invention provides a method to induce caspase-2 and caspase-3 activity to cleave poly (ADP-ribose) polymerase and fragment nuclear DNA in a cell by co-expression of a polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>.

10

Caspases are members of the protease family, the mammalian homologs of the *Caenorhabditis elegans* death gene ced-3, which are required for mammalian apoptosis. Increased levels of caspase-2 and caspase-3 have been linked to apoptosis.

15 The caspases are cysteine aspartases that cleave their substrates at aspartate residues. To activate caspases, they need to be cleaved at aspartate residues and to form active heterodimers.

20 This invention provides a method to inhibit NF- $\kappa$ B activation in a cell with a polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>.

NF- $\kappa$ B is a primary transcription factor which is activated by  
25 external stimuli, and translocated to the nucleus where it binds to DNA and regulates gene transcription. In rat Schwann cells, the binding of nerve growth factor to p75<sup>NTR</sup> neurotrophin receptor, induces the activation of NF- $\kappa$ B in the absence of tyrosine kinase receptor A, and led to cell  
30 survival. NF- $\kappa$ B regulates the gene expression of various proteins including cell surface molecules and cytokines.

This invention provides a method to detect a neurodegenerative disease in a subject by detecting  
35 expression levels of a polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>. In an embodiment of the above described

method to detect a neurodegenerative disease in a subject by detecting expression levels of a polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>, wherein the subject is a mammal. In another embodiment of the above described method  
5 to detect a neurodegenerative disease in a subject by detecting expression levels of a polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup> wherein the mammal subject is mouse, rat or human.

10 This invention provides a transgenic nonhuman mammal which comprises an isolated nucleic acid, encoding a human HGR74 protein, which is a DNA molecule. In an embodiment of the above described transgenic nonhuman mammal, the DNA encoding a human HGR74 protein is operatively linked to tissue  
15 specific regulatory elements.

This invention provides a method of determining physiological effects of expressing varying levels of a human HGR74 protein in a transgenic nonhuman mammal which comprises producing a  
20 panel of transgenic nonhuman mammal, each nonhuman mammal expressing a different amount of human HGR74 protein.

This invention provides a method of producing the isolated human HGR74 protein into a suitable vector which comprises:  
25 (a) inserting a nucleic acid molecule encoding a human HGR74 protein into a suitable vector; (b) introducing the resulting vector into a suitable host cell; (c) selecting the introduced host cell for the expression of the human HGR74 protein; (d) culturing the selected cell to produce the human  
30 HGR74 protein; and (e) recovering the human HGR74 protein produced.

This invention provides a method of inducing apoptosis of cells in a subject comprising administering to the subject  
35 the purified human HGR74 protein in an amount effective to induce apoptosis. In an embodiment of the above described



method of inducing apoptosis of cells in a subject comprising administering to the subject the purified human HGR74 in an amount effective to induce apoptosis, the subject is a mammal. In another embodiment of the above-described method of inducing apoptosis of cells in a subject, the subject is a mouse, rat or human.

This invention provides a pharmaceutical composition comprising a purified human HGR74 protein and a pharmaceutically acceptable carrier.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a subject with an appropriate amount of the compound; and (b) measuring the expression level of human HGR74 protein gene and p75<sup>NTR</sup> gene in the subject, an increase of the expression levels of human HGR74 protein gene and p75<sup>NTR</sup> gene indicating that the compound is an apoptosis inducing compound. In an embodiment of the above described method for identifying an apoptosis inducing compound comprising: a) contacting a subject with an appropriate amount of the compound; and (b) measuring the expression level of human HGR74 protein gene and p75<sup>NTR</sup> gene in the subject, an increase of the expression levels of human HGR74 protein gene and p75<sup>NTR</sup> gene indicating that the compound is an apoptosis inducing compound, wherein the subject is a mammal. In an embodiment of the above-described method of identifying an apoptosis inducing compound, wherein the mammal subject is a mouse, rat or human.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a cell with an appropriate amount of the compound; and (b) measuring the expression level of human HGR74 gene and p75<sup>NTR</sup> gene in the cell, an increase of the expression levels of human HGR74 protein gene and p75<sup>NTR</sup> gene indicating that the compound is an apoptosis inducing compound.

This invention provides a method for screening cDNA libraries human HGR74 sequence using a yeast two-hybrid system using a p75<sup>NTR</sup> intracellular domain as a target. In an embodiment of the above described method for screening cDNA libraries human HGR74 sequence using a yeast two-hybrid system using a p75<sup>NTR</sup> intracellular domain as a target, where the cDNA library is mammalian. In an embodiment of the above described method for screening cDNA libraries human HGR74 sequence using a yeast two-hybrid system using a p75<sup>NTR</sup> intracellular domain as a target, where the cDNA library is mammalian and where the mammalian cDNA library is derived from rat, mouse or human cDNA libraries. In another embodiment of the above described method for screening cDNA libraries human HGR74 sequence using a yeast two-hybrid system using a p75<sup>NTR</sup> intracellular domain as a target, where the p75<sup>NTR</sup> intracellular domain target is mammalian. In an embodiment of the above described method for screening cDNA libraries human HGR74 sequence using a yeast two-hybrid system using a p75<sup>NTR</sup> intracellular domain as a target, where the p75<sup>NTR</sup> intracellular domain target is a rat, mouse or human p75<sup>NTR</sup> intracellular domain target.

This invention provides a method to induce caspase-2 and caspase-3 activity to cleave poly (ADP-ribose) polymerase and fragment nuclear DNA in a cell by co-expression of human HGR74 protein and p75<sup>NTR</sup>.

This invention provides a method to inhibit NF- $\kappa$ B activation in a cell with human HGR74 protein and p75<sup>NTR</sup>.

30

This invention provides a method to detect a neurodegenerative disease in a subject by detecting expression levels of polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>. In an embodiment of the above described method to detect a neurodegenerative disease in a subject by detecting expression levels of polypeptide capable

of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>, wherein the subject is a mammal. In another embodiment of the above described method to detect a neurodegenerative disease in a subject by detecting expression levels of polypeptide capable of binding  
5 a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>, wherein the subject is a mammal wherein the mammal is human.

This invention provides a method of identifying a compound, which is an apoptosis inhibitor, said compound is capable of  
10 inhibiting specific binding between a polypeptide capable of binding a p75<sup>NTR</sup> receptor and p75<sup>NTR</sup> receptor, so as to prevent apoptosis which comprises: (a) contacting the polypeptide capable of binding a p75<sup>NTR</sup> receptor with a plurality of compounds under conditions permitting binding between a known  
15 compound previously shown to be able to displace the polypeptide capable of binding a p75<sup>NTR</sup> receptor and the p75<sup>NTR</sup> receptor and the bound p75<sup>NTR</sup> receptor to form a complex; and (b) detecting the displaced polypeptide capable of binding a p75<sup>NTR</sup> receptor or the complex formed in step (a), wherein the  
20 displacement indicates that the compound is capable of inhibiting specific binding between the polypeptide capable of binding a p75<sup>NTR</sup> receptor and the p75<sup>NTR</sup> receptor. In another embodiment of the above described method, wherein the inhibition of specific binding between the polypeptide  
25 capable of binding a p75<sup>NTR</sup> receptor and the p75<sup>NTR</sup> receptor affects the transcription activity of a reporter gene. In a further embodiment of the above described method, wherein step (b) the displaced polypeptide capable of binding a p75<sup>NTR</sup> receptor or the complex is detected by comparing the  
30 transcription activity of a reporter gene before and after the contacting with the compound in step (a), where a change of the activity indicates that the specific binding between the polypeptide capable of binding a p75<sup>NTR</sup> receptor and the p75<sup>NTR</sup> receptor is inhibited and the polypeptide capable of  
35 binding a p75<sup>NTR</sup> receptor is displaced. In an embodiment of the above described method, wherein the p75<sup>NTR</sup> receptor is

bound to a solid support. In a further embodiment of the above described method, wherein the compound is bound to a solid support. In an embodiment of the above described method, wherein the compound comprises an antibody, an inorganic compound, an organic compound, a peptide, a peptidomimetic compound, a polypeptide or a protein. In an embodiment of the above described method, wherein the contacting of step (a) is in vitro. In a further embodiment of the above method, wherein the contacting of step (a) is in vivo. In an embodiment of the above method, wherein the contacting of step (a) is in a yeast cell. In an embodiment of the above method, wherein the contacting or step (a) is in a mammalian cell. In an embodiment of the above method, wherein the polypeptide capable of binding a p75<sup>NTR</sup> receptor is a cell surface receptor. In an embodiment of the above method, wherein the cell-surface receptor is the p75 receptor.

As used herein, the "transcription activity of a reporter gene" means that the expression level of the reporter gene will be altered from the level observed when the signal-transducing protein and the cytoplasmic protein are bound. One can also identify the compound by detecting other biological functions dependent on the binding between the signal-transducing protein and the cytoplasmic protein. Examples of reporter genes are numerous and well-known in the art, including, but not limited to, histidine resistant genes, ampicillin resistant genes,  $\beta$ -galactosidase gene.

Further the cytoplasmic protein may be bound to a solid support. Also the compound may be bound to a solid support and comprises an antibody, an inorganic compound, an organic compound, a peptide, a peptidomimetic compound, a polypeptide or a protein.

35

An example of the method is provided infra. One can identify

a compound capable of inhibiting specific binding between the signal-transducing protein and the cytoplasmic protein using direct methods of detection such as immuno-precipitation of the cytoplasmic protein and the compound bound to a  
5 detectable marker. Further, one could use indirect methods of detection that would detect the increase or decrease in levels of gene expression. As discussed infra, one could construct synthetic peptides fused to a LexA DNA binding domain. These constructs would be transformed into the L40-  
10 strain with an appropriate cell line having an appropriate reporter gene. One could then detect whether inhibition had occurred by detecting the levels of expression of the reporter gene. In order to detect the expression levels of the reporter gene, one skilled in the art could employ a  
15 variety of well-known methods, e.g. two-hybrid systems in yeast, mammals or other cells.

Further, the contacting of step (a) may be in vitro, in vivo, and specifically in an appropriate cell, e.g. yeast cell or  
20 mammalian cell. Examples of mammalian cells include, but not limited to, the mouse fibroblast cell NIH 3T3, CHO cells, HeLa cells, Ltk<sup>-</sup> cells, Cos cells, etc.

Other suitable cells include, but are not limited to,  
25 prokaryotic or eukaryotic cells, e.g. bacterial cells (including gram positive cells), fungal cells, insect cells, and other animals cells.

This invention provides a method of identifying a compound,  
30 which is an apoptosis inhibitor, said compound is capable of inhibiting specific binding between human HGR74 protein and p75<sup>NTR</sup> receptor, so as to prevent apoptosis which comprises:  
(a) contacting the human HGR74 protein with a plurality of compounds under conditions permitting binding between a known  
35 compound previously shown to be able to displace the human HGR74 protein and the p75<sup>NTR</sup> receptor and the bound p75<sup>NTR</sup>

receptor to form a complex; and (b) detecting the displaced human HGR74 protein or the complex formed in step (a), wherein the displacement indicates that the compound is capable of inhibiting specific binding between the human HGR74 protein and the p75<sup>NTR</sup> receptor. In an embodiment of the above described method, wherein the inhibition of specific binding between the human HGR74 protein and the p75<sup>NTR</sup> receptor affects the transcription activity of a reporter gene. In a further embodiment of the above described method, wherein step (b) the displaced human HGR74 protein or the complex is detected by comparing the transcription activity of a reporter gene before and after the contacting with the compound in step (a), where a change of the activity indicates that the specific binding between the human HGR74 protein and the p75<sup>NTR</sup> receptor is inhibited and the human HGR74 protein is displaced. In an embodiment of the above described method, wherein the p75<sup>NTR</sup> receptor is bound to a solid support. In a further embodiment of the above described method, wherein the compound is bound to a solid support. In an embodiment of the above described method, wherein the compound comprises an antibody, an inorganic compound, an organic compound, a peptide, a peptidomimetic compound, a polypeptide or a protein. In an embodiment of the above described method, wherein the contacting of step (a) is in vitro. In a further embodiment of the above method, wherein the contacting of step (a) is in vivo. In an embodiment of the above method, wherein the contacting of step (a) is in a yeast cell. In an embodiment of the above method, wherein the contacting or step (a) is in a mammalian cell. In an embodiment of the above method, wherein the human HGR74 protein is a cell surface receptor. In an embodiment of the above method, wherein the cell-surface receptor is the p75 receptor.

As used herein, the "transcription activity of a reporter gene" means that the expression level of the reporter gene

will be altered from the level observed when the signal-transducing protein and the cytoplasmic protein are bound. One can also identify the compound by detecting other biological functions dependent on the binding between the  
5 signal-transducing protein and the cytoplasmic protein. Examples of reporter genes are numerous and well-known in the art, including, but not limited to, histidine resistant genes, ampicillin resistant genes,  $\beta$ -galactosidase gene.

10 Further the cytoplasmic protein may be bound to a solid support. Also the compound may be bound to a solid support and comprises an antibody, an inorganic compound, an organic compound, a peptide, a peptidomimetic compound, a polypeptide or a protein.

15

An example of the method is provided infra. One can identify a compound capable of inhibiting specific binding between the signal-transducing protein and the cytoplasmic protein using direct methods of detection such as immuno-precipitation of  
20 the cytoplasmic protein and the compound bound to a detectable marker. Further, one could use indirect methods of detection that would detect the increase or decrease in levels of gene expression. As discussed infra, one could construct synthetic peptides fused to a LexA DNA binding  
25 domain. These constructs would be transformed into the L40-strain with an appropriate cell line having an appropriate reporter gene. One could then detect whether inhibition had occurred by detecting the levels of expression of the reporter gene. In order to detect the expression levels of  
30 the reporter gene, one skilled in the art could employ a variety of well-known methods, e.g. two-hybrid systems in yeast, mammals or other cells.

Further, the contacting of step (a) may be in vitro, in vivo,  
35 and specifically in an appropriate cell, e.g. yeast cell or mammalian cell. Examples of mammalian cells include, but not

limited to, the mouse fibroblast cell NIH 3T3, CHO cells, HeLa cells, Ltk<sup>-</sup> cells, Cos cells, etc.

Other suitable cells include, but are not limited to, 5 prokaryotic or eukaryotic cells, e.g. bacterial cells (including gram positive cells), fungal cells, insect cells, and other animals cells.

In order to facilitate an understanding of the material which 10 follows, certain frequently occurring methods and/or terms are best described in Sambrook, et al., 1989.

This invention provides an isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable 15 of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the N-terminal 1-40 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(41-124), and the NADE N(41-124) induces apoptosis in the 20 presence of p75<sup>NTR</sup>.

This invention also provides an isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, 25 designated neurotrophin associated cell death executor protein (NADE), wherein the C-terminal 72-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(1-71), and the NADE N(1-71) induces apoptosis in the presence of p75<sup>NTR</sup> and in the absence 30 of p75<sup>NTR</sup>.

This invention further provides an isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, 35 designated neurotrophin associated cell death executor protein (NADE), wherein the N-terminal 1-40 amino acids and the C-terminal 72-124 amino acids of wild type NADE



polypeptide have been deleted and the deletion mutant is designated NADE N(41-71), and the NADE N(41-71) induces apoptosis in the presence of p75<sup>NTR</sup> and in the absence of p75<sup>NTR</sup>.

5

This invention provides an isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the C-terminal 121-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(1-120) and the NADE N(1-120) induces apoptosis in the presence of p75<sup>NTR</sup>.

15 This invention also provides an isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the C-terminal 113-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(1-112) and the NADE N(1-112) induces apoptosis in the presence of p75<sup>NTR</sup>.

This invention further provides an isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the C-terminal 101-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(1-100) and the NADE N(1-100) induces apoptosis in the presence of p75<sup>NTR</sup> and in the absence of p75<sup>NTR</sup>.

This invention further provides an isolated nucleic acid molecule encoding a mutation of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE),

wherein the point mutation results in Ala at amino acid position 99 for Leu at amino acid position of wild type NADE polypeptide, wherein the substitution mutant polypeptide is designated NADE N(L99A) and the NADE N(L99A) induces 5 apoptosis in the presence of p75<sup>NTR</sup>.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and 10 results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

## Experimental Details

### **Results and Discussions**

The p75<sup>NTR</sup> is the first-isolated neurotrophin receptor and the 5 member of TNFR (tumor necrosis factor receptor) family (7, 8). However, its functional role and signaling pathway has remained largely unclear (9). The existence of p75<sup>NTR</sup>ICD binding proteins have been implicated since p75<sup>NTR</sup>ICD does not have a typical biochemical motif except a C-terminal region 10 well conserved to a type 2 death domain (10). Recently, it has been reported that TRAF6 is involved in p75<sup>NTR</sup>-mediated signal transduction(11). To further identify the p75<sup>NTR</sup>ICD binding proteins, we screened the mouse cDNA libraries by yeast two-hybrid system using a rat p75<sup>NTR</sup>ICD as a target and 15 one of positive clones was identified as a p75<sup>NTR</sup>-associated cell death executor, NADE.

NADE consists of 124 amino acids and its molecular weight is calculated to 14,532 dalton. NADE is a hydrophilic and 20 acidic protein, and the estimated pI value is 5.97. A BLAST search revealed that NADE has significant homology to a known human protein HGR74(4) (Fig. 1a), and does not have a significant motif except the leucine rich nuclear export signal (NES) (5) (Fig. 1b) and ubiquitination sequences (6) 25 (Fig. 1c) HGR74 was previously reported as an abundant mRNA expressed in human ovarian granulosa cells, however, its functional role is still unknown. The homology of these two proteins except the asparagine rich stretch (a. a. 36-48) of NADE is 92.8%, therefore we conclude that HGR74 is a human 30 homolog of mouse NADE.

Northern blot analysis is revealed that NADE mRNA (1.3 kbp) is found highest in several tissues including brain, heart, and lung (Fig. 1d). We could also detect a low level of mRNA 35 expression in stomach, small intestine, and muscle by a long exposure (data not shown). But there was no expression in liver. The additional large band (3.0 kbp) was also observed in testis, suggesting the existence of the alternative

splicing form. The endogenous NADE protein was also confirmed in human neuroblastoma cell line, SK-N-MC by immunoprecipitation using the anti-NADE antibody (Fig. 1e). Interestingly, in SK-N-MC, PC12 and PCNA cells, NADE protein  
5 can be detected only in the presence of the ubiquitin inhibitor such as ALLN, suggesting that NADE is modified by ubiquitin conjugating system leading to subsequent degradation by the proteasome. The molecular size of NADE is estimated to 22 kDa by the SDS-PAGE, and this size seems to  
10 be slightly larger than the molecular weight predicted from nucleotide sequence. But the gap of molecular size might be caused by its low pI value or post-translational modification in a potential prenylation site (Fig. 1a). The overexpressed NADE protein in 293T cells showed the two bands, 22 kDa and 44  
15 kDa in SDS-PAGE under the reduced condition at 100 mM dithiothreitol (Fig. 1f). To clarify this question, two NADE mutants were constructed and expressed in 293T cells. Since NADE has two cysteine residues at sequence positions 102 and 121, we replaced the each cysteine with the serine residue.  
20 Western blot analysis revealed that the molecular weight of muNADE (Cys121Ser) is identical to a wild type, on the other hand, muNADE (Cys102Ser) showed the only smaller band of 22 kDa (Fig. 1g). These results strongly suggested that NADE can heterodimerize by the disulfide bond at the Cys102, and  
25 resulted in the 44 kDa band.

*In vitro*-translated mouse NADE protein and *E. coli*-expressed GST-p75<sup>NTR</sup>ICD fusion protein were used for *in vitro* GST pull down assay. In this assay, the NADE protein showed the  
30 strong binding activity to GST-P p75<sup>NTR</sup>ICD (Fig. 2a). To investigate the *in vivo* binding activity, the Myc-tagged NADE and p75<sup>NTR</sup> were co-expressed in 293T cells and subjected to the co-immunoprecipitation experiment. The results clearly showed that NADE could bind to a full length of p75<sup>NTR</sup> *in vivo*  
35 very strongly (Fig. 2b) and the recruitment of NADE protein to p75<sup>NTR</sup>ICD was detected in a dose dependent of NGF (Fig. 2c), suggesting that NADE protein is a putative signal

transducing protein interacting with p75<sup>NTR</sup>ICD. Furthermore, our mapping studies revealed that NADE protein interacts with the cell death domain (amino acid residues 338-393) which is identical among mouse, rat and human (data not shown). Since 5 TRAF6 binds a conserved juxtamembrane region (11), it is unlikely that NADE protein inhibits TRAF6 binding to p75<sup>NTR</sup>. It has been speculated that the polymerization of p75<sup>NTR</sup> is important for its signal transduction similar to the another members of TNFR family. For example, TNFRI (12), CD40 (13), 10 and Fas (14) are formed the trimer through the binding of each trimer ligands to extracellular domain. However, there was no previous report for p75<sup>NTR</sup> in same manner (15). It may be possible that the dimer formation of p75<sup>NTR</sup> occurs through the binding of NADE dimer to its intracellular domain.

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To investigate the functional role of NADE protein, NADE and p75<sup>NTR</sup> were co-transfected in 293T cells. The results showed that the co-transfected 293T cells were detached from the dish and aggregated 48 hours later (Fig. 3a). However, 293T 20 cells transfected with the control plasmid DNAs showed no significant differences (Fig. 3a), implicating that this morphological change is caused by apoptosis. We further examined the TUNEL assay (TdT-mediated dUTP-biotin nick end labeling assay) (16) as well as the DNA fragmentation test on 25 these cells. On the TUNEL assay, the significant increase of dying cell was detected only in co-transfected cells (Fig. 3b) and the value of the positive cell percentage (38%) was consistent with the transfection efficiency by the calcium-phosphate method. Furthermore, the DNA fragmentation was 30 detected in only the co-transfected 293T cells (Fig. 3c). From these results, we conclude that the co-expression of NADE and p75<sup>NTR</sup> induced apoptosis in 293T cells.

Although NADE protein is recruited to the cytoplasmic region 35 of p75<sup>NTR</sup> in a ligand-dependent manner, NGF-dependent cell death was not clearly detected in the co-transfected 293T cells in the presence of NGF (100 ng/ml) (data not shown), suggesting that NADE protein may function in the p75<sup>NTR</sup>-

mediated cell death machinery to transduce the downstream signal to apoptosis independent on NGF.

To further investigate the physiological function of NADE  
5 protein, we checked the transcription factor kappa B (NF-kB),  
Caspase-2, and Caspase-3 activities in 293T cells co-  
transfected with NADE and p75<sup>NTR</sup>. NF-kB is activated by  
external stimuli, and translocated to the nucleus where it  
binds to DNA and regulates gene transcription (17). In rat  
10 Schwann cells, the binding of NGF to p75<sup>NTR</sup> induces the  
activation of NF-kB with independent manner of TrkA (18)  
leading to the cell survival and TRAF6 may be a component of  
NGF-mediated NF-kB activation (11). In contrast, expression  
of NADE protein significantly suppressed the NF-kB activity  
15 in a dose dependent manner, but this effect was not markedly  
co-operative with p75<sup>NTR</sup> expression (Fig. 3d) as well as NGF-  
dependent manner (data not shown), implicating that  
p75<sup>NTR</sup>/NADE-induced apoptosis may not be due to only the  
suppression of NF-kB activity but also the regulation of  
20 unknown signal molecules since NF-kB suppression by NADE  
protein alone could not induce apoptosis. It has been  
reported that suppression of NF-kB activity increases cell  
death in PC12 cells expressing p75<sup>NTR</sup> (19, 20). NADE protein  
may play a key role in the downregulation of NF-kB activity  
25 and ultimately lead to apoptosis in neuronal cells expressing  
p75<sup>NTR</sup>.

In many cases of apoptosis, the elevation of Caspase-3  
activity was observed (21, 22, 23, 24). This protease  
30 normally exists in cytosol of cells as 32 kDa precursor that  
is proteolytically activated into a 20 kDa and a 10 kDa  
heterodimer when cells are signaled to undergo apoptosis in  
response to serum withdrawal, activation of Fas, treatment  
with ionization, and a variety of pharmacological agents  
35 (25). Western blot analysis revealed that Caspase-2 and  
Caspase-3 were significantly processed only in 293T cells co-  
transfected with NADE and p75<sup>NTR</sup> (Fig. 3e). Moreover, PARP  
(poly (ADP-ribose) polymerase) which is a substrate for both

Caspase-2 and Caspase-3 were partially cleaved, indicating that these Caspases are involved in apoptosis mediated by p75<sup>NTR</sup>/NADE signal transduction

5 To investigate whether NES sequences (5) contained in NADE (Fig. 4a) have the capability to export a protein from the nucleus to the cytosol, we performed the transient expression in 293T cells using a series of NADE mutants. The results indicated that NADE proteins with NES sequences localize in  
10 the cytoplasmic region (Fig. 4, lower panels of b, upper panels of c and d), but NADE proteins with NES mutations express in the nucleus (Fig. 4, lower panel of c and d). These data support the hypothesis that NADE protein can be exported from the nucleus to the cytosol and may be post-  
15 translationally modified as a prenylated protein to promote and regulate p75<sup>NTR</sup>/NADE physiological interaction.

The signal cascade mediated by p75<sup>NTR</sup> has been enigmatic for a long time. But the recent growing evidences indicate that,  
20 not like other members of TNFR family, p75<sup>NTR</sup> can bifunctionally mediate signals to induce and inhibit apoptosis (26, 27). Our results strongly supported that NADE is a putative signal transducer for p75<sup>NTR</sup>-mediated apoptosis. Although NADE can mediate apoptosis cooperative with p75<sup>NTR</sup>,  
25 it is possible that NADE may be a signal adaptor molecule to interact with another effector molecules in p75<sup>NTR</sup>-mediated signal transduction. More importantly, since NADE has nuclear export signal (NES) as well as ubiquitination sequence, NADE may be tightly controlled by the  
30 ubiquitin/proteasome to shuttle another molecule from the nucleus to the cytoplasm, implicating that NADE is a very important protein for turnover of regulator gene such as the cell cycle-related proteins. Further investigation under physiological condition will give us more insight to better  
35 understand the mechanisms by which NADE can induce apoptosis together with p75<sup>NTR</sup> expression.

## Methods

**Isolation of p75<sup>NTR</sup>-associated cell death executor (NADE) by yeast two-hybrid system.**

In order to isolate cDNA encoding p75<sup>NTR</sup>-associated proteins, we used yeast two-hybrid system, originally developed by Fields and Song (28). We used the cytosolic domain of rat p75<sup>NTR</sup> cDNA corresponding to amino acids 338-396 (representing the cytosolic domain of the protein from the transmembrane domain to the C-terminus of the protein) as a target. This portion of p75<sup>NTR</sup> cDNA was PCR-engineered into the yeast expression plasmid pBTM116 in-frame with sequences encoding the LexA DNA-binding domain (29). This plasmid was then introduced into L40 cells [a, his3, trp1, leu2, ade2, lys2: (lexAop)<sup>4</sup>-HIS3, URA3: (lexAop)<sup>8</sup>-lacZ] which contain histidine synthetase (HIS3) and b-galactosidase (lacZ) reporter genes under the control of lexA operators (29). After confirming the expression of LexA-p75<sup>NTR</sup> (338-396) protein by immunoblotting using an antiserum specific for LexA, a mouse embryo pVP16 cDNA libraries were then introduced into these LexA/p75<sup>NTR</sup>-expressing cells by a high efficiency LiOAc transformation method (30, 31, 32). From a screen of 5 x 10<sup>7</sup> transformants, an initial set of 672 His<sup>+</sup> colonies were identified. These 672 clones were then tested by a  $\beta$ -galactosidase colorimetric assay (33), utilizing the lacZ reporter gene under the control of 8 lexA operators, thus narrowing down the pool of candidate clones to 181. These 181 candidates were then "cured" of their LexA/p75<sup>NTR</sup>-encoding plasmids by growth in tryptophan containing media, and mated with a panel of Mata-type yeast strain NA87-11A [a, leu2, his3, trp1, pho3, pho5] into which we had introduced various control plasmids that produce LexA fusion proteins, including LexA/p75<sup>NTR</sup>, LexA/Ras, Lex/CD40, LexA/Fas, and LexA/lamin. Among the 181 candidate clones, 1 clone specifically reacted with the LexA/p75<sup>NTR</sup> protein was chosen for further analysis. This mouse cDNA clone No. 59 has insert sizes of 450 bp. Because of its ability to induce cell death with expression of p75<sup>NTR</sup>, we have named this protein, NADE (p75<sup>NTR</sup>-associated cell death executor).



**DNA construction.**

A full length mouse NADE cDNA was constructed on pBluescript II vector by the ligation of the partial NADE cDNA (7-524) and 5'-RACE product. PCR cloning techniques were used to replace the stop codon and add the 5' *XhoI* site and 3' *BamHI* site of a full length NADE cDNA. pcDNA3.1(-)Myc-HisA/NADE was constructed by insertion of a full length NADE cDNA to *XhoI*-*BamHI* site of pcDNA3.1(-)Myc-HisA (Invitrogen). Human NADE cDNA was amplified using a Jurkat T cell cDNA library and  
10 cloned to pcDNA3.1(-)Myc-HisA pcDNA3/rat p75<sup>NTR</sup> was constructed by insertion of a full length rat p75<sup>NTR</sup> cDNA to *EcoRI* site of pcDNA3 (Invitrogen). pGEX4T-1/rat p75<sup>NTR</sup>ICD was constructed by insertion of amplified rat p75<sup>NTR</sup>ICD (a. a. 338-396) to pGEX4T-1 (Pharmacia). Mutant NADE expression  
15 plasmids, pcDNA3.1(-)Myc-HisA/muNADE (Cys102Ser) and pcDNA3.1(-)Myc-HisA/muNADE(Cys121Ser), were constructed by PCR-based site-direct mutagenesis methods (29). pELAM-Lu for luciferase reporter assay was constructed by insertion of NF- $\kappa$ B binding site of E-selectin promoter region (-730 - 52)  
20 to pGL3-Basic *SacI*-*BglIII* site. Expression plasmids of GFP-fused NADE proteins were made following: The cDNA of GFP was cloned into *NheI*-*XhoI*-cut pcDNA3.1-mouse NADE as a PCR product amplified with the primers 5'-CTAGCTAGCATCATGGTGAGCAAGGGCGAG-3' and 5'-CCGCTCGAGTCTTGTACAGCTCGTCCAT-3' using pEGFP-N2 (Clontech) as  
25 a template. The deletion mutants delta 101-124-GFP and delta 91-124-GFP were constructed by inserting an *XhoI*-*BamHI*-cut PCR fragment generated with Expand high fidelity Taq polimerase (Boehringer Mannheim) into *XhoI*-*BamHI*-cut  
30 pcDNA3.1-GFP using the primers  
5'-ATCCTCGAGCGATCATGGCCAATGTCCAC-3' (sense),  
5'-ATCGGATCCTCTCAGCTGTAGCTCCCT-3' (antisense) and  
5'-ATCGGATCCGATCTCTCATCTCCTC-3' (antisense).

35 The mutagenic primers  
(5'-AAAGCTTAGGGAGGCACAGCTGAGAAA-3',  
5'-TTTCTCAGCTGTGCCTCCCTAAGCTTT-3',

5"-ATCCGGAGAAAGGCTAGGGAGGCACA-3",  
and 5"-TGTGCCTCCCTAGCCTTTCTCCGGAT-3")

were used to obtain L97A-GFP and L94, 97A-GFP in which Leu94 and Leu97 are replaced with Ala. In all constructs, 5 mutations were verified by sequencing.

**Northern blot analysis.** 400 ng of NADE cDNA fragments (nt. 5-510) were labeled by 50  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]dCTP and used as a probe. Each 10  $\mu$ g of total mRNA extracted from mouse various  
10 tissues were transferred on membranes and they were hybridized with a NADE probe for 2 hours at 68 °C using a express hybrid buffer (Clontech) and washed with 2 x SSC, 0.05 % SDS for 5 times, and 0.1 x SSC, 0.1 % SDS for 1 time.

**15 Antibodies.** The polyclonal anti-NADE antibody was prepared by immunization of GST-mouse NADE fusion protein into the rabbit. The NADE specific antibody was affinity purified by antigen coupled Sepharose 4B. The polyclonal anti-rat p75<sup>NTR</sup> was kindly gifted from Dr. M. V. Chao. The monoclonal anti-  
20 Myc antibody (9E10) was purchased from BIOMOL. The polyclonal anti-Caspase-3 antibody (H-277) was purchased from Santa Cruz Biotechnology. The polyclonal Caspase-2 antibody was kindly gifted from Dr. Lloyd A. Greene. HRP conjugated anti-rabbit IgG was purchased from Bio-Rad.

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**Immunoprecipitation and immunoblotting.** In Fig. 1e, 150  $\mu$ g/ml of ALLN (N-Acetyl-Leu-Leu-Norleucinal) treated SK-N-MC cells ( $1 \times 10^7$ ) were lysed in 0.5 ml of RIPA buffer. The supernatant of centrifuge (100,000 x g) was mixed with 1  $\mu$ g  
30 of polyclonal anti-NADE antibody coupled Sepharose 4B, and incubated for 4 hours at 4 °C. After washing, the gels were boiled by 30  $\mu$ l of SDS-PAGE sampling buffer and subjected to 12.5 % of SDS-PAGE. Immunoblotting was performed by polyclonal anti-NADE antibody (2  $\mu$ g/ml). In Fig. 1f, 10  $\mu$ g  
35 of cell lysate extracted from each transfected 293T cells were used for the detection of NADE by immunoblotting.

**Transfection and protein expression in 293T cell.** In Fig. 1f, 293T cells ( $2 \times 10^6$ ) were transfected by 10  $\mu$ g of pcDNA3.1(-)Myc-HisA/NADE, pcDNA3.1(-)Myc-HisA/muNADE (Cys102Ser), or pcDNA3.1(-)Myc-HisA/muNADE (Cys121Ser) by 5 calcium-phosphate method. In Fig. 2 b, 3 a, b, c, e, 293T cells ( $2 \times 10^6$ ) were transfected by 20  $\mu$ g of pcDNA3.1(-) Myc-HisA, 10  $\mu$ g of pcDNA3/rat p75<sup>NTR</sup> and 10  $\mu$ g of pcDNA3.1(-) Myc-HisA, 10  $\mu$ g of pcDNA3.1(-)Myc-HisA NADE and 10  $\mu$ g of pcDNA3.1(-) Myc-HisA, or 10  $\mu$ g of pcDNA3.1(-)Myc-HisA/NADE and 10  $\mu$ g of pcDNA3 / rat p75<sup>NTR</sup>. In Fig. 2 c, 293T cells ( $2 \times 10^6$ ) were transfected by 10  $\mu$ g of pcDNA3.1(-)Myc-HisA/NADE and 10  $\mu$ g of pcDNA3/rat p75<sup>NTR</sup> in serum minus DMEM medium.

**In vitro binding assay.** 5  $\mu$ l of L-[<sup>35</sup>S] methionine labeled, and in vitro- translated NADE protein was mixed with 5  $\mu$ l of GST-rat p75<sup>NTR</sup>ICD fusion protein or GST-coupled GSH-Sepharose 4B (Pharmacia) in 100  $\mu$ l of NETN buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.2 % NP-40) for 18 hours at 4 °C. After washing, gels were boiled by 30  $\mu$ l of SDS-PAGE sampling buffer and subjected to 13.5 % SDS-PAGE. The fluorography was performed for 16 hours at -70 °C.

**In vivo binding assay.** In Fig. 2b, transfected 293T cells by were lysed in 1 ml of NETN buffer and centrifuged (100,000  $\mu$ g). The supernatants were immunoprecipitated by 2  $\mu$ g of anti-Myc antibody coupled Protein G Sepharose 4B (Pharmacia) for 2 hours at 4 °C. Following the 5 times washing, gels were subjected to 7.5 % SDS-PAGE, and Western blot analysis by rabbit polyclonal anti-p75<sup>NTR</sup> antibody.

30

**Interaction of NADE with p75<sup>NTR</sup> dependent on NGF ligation.** After co-transfection, cells were incubated in DMEM medium containing various NGF. After 12 hours later, the interaction activity between NADE and p75<sup>NTR</sup> were checked by in vivo binding assay.

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**TUNEL assay.** MEBSTAIN Apoptosis kit direct (MIC) was used for TUNEL assay and the assay was done according to the company instruction. The stained cells were analyzed by FACSCalibur flow cytometer (Becton Dickinson).

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**DNA fragmentation assay.** Transfected 293T cells were lysed in 350  $\mu$ l of 10 mM EDTA and 0.5 % SDS for 10 minutes at room temperature. After adding 100  $\mu$ l of 5 M NaCl, the aliquot was incubated for 18 hours at 4 °C and centrifuged (12,000 x 10 g). The supernatants were treated by 1 mg/ml of RNase A and 50 ng/ml of Proteinase K for 2 hours at 42 °C. After the phenol-chloroform extraction, the DNAs were precipitated by 70 % ethanol, and dissolved in 30  $\mu$ l of H<sub>2</sub>O. 5  $\mu$ l of samples were subjected to the 1.5 % agarose gel electrophoresis.

15

**Measurement of NF- $\kappa$ B activity.** Dual-Luciferase Reporter Assay System (Promega) was used for measurement of NF- $\kappa$ B activity. 293T cells ( $4 \times 10^5$ ) were transfected with 1.5  $\mu$ g of pELAM-luc reporter plasmid, 0.1  $\mu$ g of pRL-TK, 0.7  $\mu$ g of pcDNA3 rat p75<sup>NTR</sup>, 0.3  $\mu$ g or 2.8  $\mu$ g of pcDNA3.1(-) Myc-HisA/NADE and enough pcDNA3.1(-) Myc-His a control plasmid to give 5.1  $\mu$ g of total DNA. Luciferase activities were determined 24 hours after transfection and normalized on the basis of pRL-TK expression levels. The luciferase activities were measured by 25 Turner Designs Luminometer Model TD20/20 (Promega).

#### **Confocal laser microscopy**

Transient transfections were carried out using the calcium phosphate precipitation method. 293T cells ( $3 \times 10^5$ ) on a cover 30 glass were transiently transfected with 3.0  $\mu$ g of DNA. After 12-24 hours, cells were fixed with 4 % paraformaldehyde and stained with TO-PRO-3 Iodide (Molecular Probes, Inc.) to visualize the nucleus. The subcellular distribution of GFP fusion proteins was examined using confocal laser microscopy 35 (Carl Zeiss LSM510).

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Second Series of Experiments**Structure-function Analysis of NADE, Which Mediates NGF-induced Apoptosis and Nuclear Factor- $\kappa$ B Suppression****SUMMARY**

5        Low-affinity neurotrophin receptor p75NTR can mediate apoptosis of neural cells by nerve growth factor (NGF). We recently identified p75NTR-associated protein, NADE (p75NTR-associated cell death executor) and demonstrated that NADE induces apoptosis after interacting with p75NTR-intracellular domain (ICD) in response to NGF in 293T, 10        PC12 and nnr5 cells (Mukai, et. al., (2000) *J. Biol. Chem.* 275, 17566-17570). To further gain insight into the functional and structural features of NADE protein, we performed extensive mutational analysis on NADE.

15        Truncation of minimal region comprising residue 41 to 71 was sufficient to induce apoptosis, whereas this pro-apoptotic domain appeared to mediate apoptosis in NGF/p75NTR-independent manner. In contrast, deletion of N-terminal 40 residues (41-124) still remained the ability 20        of NGF-dependent apoptosis. Thus, C-terminal amino acid residues (72-112), designated regulatory domain, are essential for NGF-dependent regulation of NADE-induced apoptosis. Furthermore, the mutants with amino acid substitutions in leucine-rich nuclear export signal (NES) 25        sequences (residues 90-100), which located in regulatory domain, abolished the NADE export from nucleus to cytoplasm, dimerization, interaction with p75NTR, and NGF-dependent apoptosis. Interestingly, overexpression of NADE protein suppressed NF- $\kappa$ B activity in 293T, PC12, and 30        nnr5 cells in NGF-independent manner. In contrast, point mutant (Cys 121 $\rightarrow$ Ser) activates NF- $\kappa$ B activity as dominant negative form. Taken together, the distinct domains are involved in regulating NADE functions, such as NGF-

dependent recruitment and apoptosis, to mediate p75NTR signal transduction.

#### INTRODUCTION

Many types of mammalian cells undergo apoptosis during normal development or in response to a variety of stimuli, including DNA damage, growth factor deprivation, and abnormal expression of oncogenes or tumor suppressor genes (1-3). Apoptosis induced by these various reagents appears to be mediated by a common set of downstream elements that act as regulators and effectors of apoptotic cell death. Neurotrophins have also been shown to promote apoptosis during normal development in neural cells. In contrast to the survival function mediated via Trk receptors, neurotrophin-induced apoptosis is mediated via common neurotrophin receptor, p75NTR, which is a member of the TNF receptor superfamily (4). Pro-apoptotic role of p75NTR has been supported by the results in a variety of systems, including cultured cells as well as knockout and transgenic mice (5-7). However, the molecular mechanism of pro-apoptotic signaling involved in p75NTR is not well characterized. Recently, tumor necrosis factor receptor-associated factor (TRAF) family proteins, FAP-1, and zinc finger proteins have been reported to interact with p75NTR (ICD) (8-12). However, none of them had a direct effect on NGF-dependent apoptosis.

Recently, we identified a novel protein, named NADE, which binds to p75NTR intracellular domain (ICD) by yeast two-hybrid screening (13). Coexpression of NADE and p75NTR induced cell death in 293T cells. NGF-induced recruitment of NADE to p75NTR(ICD) was dose-dependent, and p75NTR/NADE-induced cell death required NGF but not BDNF, NT-3 or NT-4/5. Similar results were also obtained from PC12 and nnr5 cells. NADE has a consensus motif, nuclear export signal (NES), which is necessary and sufficient to mediate nuclear export of large carrier proteins (14).

Many proteins have been recently reported to be spatially controlled by their NES, including HIV-Rev (15), PKI (16) and MAPKK (17). NES-mediated intracellular transport system is a universal and conserved mechanism to control the subcellular localization of proteins in cells.

One of the key proteins that modulates the apoptotic response is NF- $\kappa$ B, a transcription factor that can protect or contribute to apoptosis. The role of NF- $\kappa$ B activation during apoptosis induced by various stimuli is still in debate; it has been suggested to have both pro-apoptotic (18) and anti-apoptotic (19, 20) properties, depending on the cell type. p75NTR has been reported to mediate NGF-induced NF- $\kappa$ B activation as anti-apoptotic signal in Schwann cells (8) and pro-apoptotic signal in oligodendrocytes (21). In contrast, inhibition of NF- $\kappa$ B activity induces cell death in response to NGF in rat schwannoma cell line (22), PC12 cells (23) or sympathetic neurons (20).

In this study, we analyze three issues relating to the functional and structural properties of mouse NADE. First, mutational analysis on NADE defined requirements for cell death in response to NGF. Second, we showed that the NADE contains a functional NES domain and that this sequence is responsible for self-association, interaction with p75NTR and induction of cell death. Finally, we have demonstrated that two distinct domain of NADE suppressed NF- $\kappa$ B activity in 293T cells.

#### EXPERIMENTAL PROCEDURES

**Constructs.** NADE (WT) (pcDNA3.1/myc-His(-)A/mNADE WT) was constructed as described previously (10). Expression vectors for mNADE deletion mutants were constructed by PCR amplification of mNADE coding sequences using oligonucleotide pairs as shown, digesting the resulting fragments with *XhoI*/*Bam*HI, and ligating the resulting

fragments into *XhoI/BamHI*-digested pcDNA3.1/*myc-His(-)* A:  
 for N (1-120),  
 FX29 (5'-ATCCTCGAGCGATCATGGCCAATGTCCAC-3') and RB360 (5'-  
 ATCGGATCCGAATTCATCATGGTGATC-3'); for N (1-112), FX29 and  
 5 RB336 (5'-ATCGGATCCGTTAGACAGCTCCCCCAT-3'); for N (1-100),  
 FX29 and RB300 (5'-ATCGGATCCTCTCAGCTGTAGCTCCCT-3'); for N  
 (1-90), FX29 and RB270 (5'-ATCGGATCCGATCTCTCTCATCTCCTC-  
 3'); for N (1-71), FX29 and RB213 (5'-  
 ATCGGATCCGTCATTCATCTGCCTGTT-3'); for N (1-60), FX29 and  
 10 RB180 (5'-ATCGGATCCGAAGTTAGGGGCAAGTCG -3); for NADE (1-  
 20), FX29 and RB60 (5'-ATCGGATCCTTCCTGTCCATTCTGCAG-3');  
 for N (41-124), FX121 (5'-  
 ATCCTCGAGACCATGCACAACCATAACCACAAC-3') and RB27; for N (81-  
 124), FX241 (5'-ATCCTCGAGACCATGGAAATGTTTCATGGAGGAG-3') and  
 15 RB27; for N (101-124), FX301 (5'-  
 ATCCTCGAGACCATGAATTGTCTACGCATCCTT-3') and RB27; for N (41-  
 71), FX121 and RB213.

Point mutants for mNADE were constructed by PCR  
 amplification of mNADE coding sequences using  
 20 oligonucleotide pairs as shown, digesting the resulting  
 PCR product with *DpnI* : for N (C121S), in which Cys-121 is  
 replaced with Ser, F-C121S (5'-ATGATGAATTCTCTTATGCCTGGA-  
 3') and R-C121C (5'-TCCAGGCATAAGAGAGAATTCATCAT-3'); for N  
 (L99A) and GFP-N (L99A), in which Leu-99 is replaced with  
 25 Ala, F-L99A (5'-AGGGAGCTACAGGCGAGAAATTGTCTA-3') and R-L99A  
 (5'-TAGACAATTTCTCGCCTGTAGCTCCCT-3'); for N (L94A, L97A,  
 L99A) and GFP- N (L94A, L97A, L99A), in which Leu-94, Leu-  
 97 and Leu-99 are replaced with Ala, F-L97A (5'-  
 AAAGCTTAGGGAGGCACAGCTGAGAAA-3'), R-L97A (5'-  
 30 TTTCTCAGCTGTGCCTCCCTAAGCTTT-3') and F-L97A, L99A (5'-  
 AGGGAGGCACAGGCGAGAAATTGTCTA-3'), R- L97A, L99A (5'-  
 TAGACAATTTCTCGCCTGTGCCTCCCT-3') and F-L94A, L97A (5'-  
 ATCCGGAGAAAGGCTAGGGAGGCACA-3'), R-L94A, L97A (5'-  
 TGTGCCTCCCTAGCCTTTCTCCGGAT-3').

Expression plasmids for green fluorescence protein (GFP)-fused mNADE proteins were made as follows: GFP cDNA was PCR-amplified from pEGFP-N2 (CLONTECH) by using the primer pair 5'-CTAGCTAGCATCATGGTGAGCAAGGGCGAG-3' and 5'-CCGCTCGAGTCTTGTACAGCTCGTCCAT-3'. The product was cloned into NheI-XhoI-digested pcDNA3.1/myc-His(-)A/mNADE. Expression plasmids for glutathione S-transferase (GST)-fused p75NTR proteins were used as described previously (22).

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**Reagents and Antibodies.** Mouse nerve growth factor (NGF) was obtained from Sigma. TO-PRO-3 iodide was obtained from Molecular Probes. The anti- $\alpha$ -NADE polyclonal antibody was prepared as described previously (10).

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**Cell culture and transfection.** 293T cells were obtained from American Type Culture Collection; PC12 and nnr5 cells were obtained from Dr. L. A. Greene (Department of pathology, Columbia University). 293T cells were maintained in DMEM supplemented with 10% FBS. PC12 and nnr5 cells were maintained in RPMI 1640 supplemented with 10% horse serum and 5% calf serum. For transfection, 293T cells ( $1.5 \times 10^6$  per 100-mm dish) were transiently transfected with 25  $\mu$ g plasmid according to the calcium phosphate method in DMEM supplemented with 10% FBS and cultured for 10 h. After withdrawing the serum, the cells were treated with 100 ng/ml NGF for 36 h.

**Subcellular localization analysis.** 293T cells were plated onto glass coverslips and transfected with GFP-containing constructs. At 24 h after transfection, cells were fixed with 3.7% paraformaldehyde, washed with PBS, and stained with TO-PRO-3 iodide to visualize the nucleus. The images of representative fields were captured on a Zeiss LSM 510 confocal laser-scanning microscope.

***In vitro binding assay.*** In vitro-translated [<sup>35</sup>S] methionine-labeled proteins were generated by using the TNT-coupled reticulocyte lysate system (Promega). Binding  
5 assay was performed as described previously (22).

***Apoptosis assay and DAPI staining,*** The transfected cells were washed with PBS, fixed in 3.7% paraformaldehyde, and stained with 50 g/ml of DAPI. By using fluorescence  
10 microscopy, the number of cells that had the nuclear morphology typical of apoptosis among at least 400 cells were counted in each sample.

***Reporter assay.*** 293T cells were plated in six well plated  
15 at 5 X 10<sup>4</sup> cells per well and transiently transfected with 5 µg of plasmids containing 0.7 µg of pELAM-luc reporter plasmid (a gift of MBL Co., Ltd) and 0.1 µg of pRL-TK as an internal control (Promega), using the calcium/phosphate method. At 24 h after transfection, the cells were washed  
20 twice with phosphate-buffered saline (PBS) and lysed in 200 µl of lysis buffer (Dual-Luciferase Reporter Assay System, Promega). Lysate (10 µl) was mixed with 50 µl of luciferase assay reagent. Luciferase activity was measured in a model LB9507 luminometer (EG and G Berthold,  
25 Germany).

***Western blot.*** After lysing the cells in lysis buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.2 % NP-40, 1 mM PMSF, 1 mM benzamidin, 50 µg/ml leupeptin, 7 µg/ml  
30 pepstatin A), we separated the lysate on 12.5 % SDS-PAGE gels. The separated proteins were then transferred to PVDF membrane (Bio-Rad). The membrane was blocked with blocking buffer (10% skim milk and 0.1% NaN<sub>3</sub> in PBS) at room temperature. Immunoreactive products were detected by

using the ENHANCE chemiluminescence system (Amersham Pharmacia).

## RESULTS

### 5 *Characterization of NADE Deletion Mutants for Apoptosis.*

To investigate the structural features of NADE required for apoptosis, we generated a series of NADE deletion mutants (Fig. 5A). In order to analyze p75NTR/NADE-mediated apoptosis, we performed DAPI staining for nuclear morphological analysis of apoptosis in addition to TUNEL assay. 293T cells were transiently transfected with pcDNA3.1myc-His/mNADE (WT) or/and pcDNA3p75NTR, treated with or without 100 ng/ml NGF. Cells cotransfected with wild-type NADE and under NGF treatment caused the typical morphological characteristics of apoptosis, including nuclear condensation and fragmentation (Fig. 6A). By contrast, single transfectants with wild-type NADE or p75NTR displayed a normal nuclear morphology similar to that with control vector. When apoptotic cells were scored based on morphological criteria, approximately 45 % of 293T cells, which were cotransfected with wild-type NADE and p75NTR, displayed apoptotic morphology in response to NGF (Fig. 6B). Similar results were obtained from TUNEL assay (data not shown).

25 To identify the region responsible for apoptosis, truncation mutants of NADE were analyzed for their ability to induce apoptosis. As shown in Fig. 7 and Table 1, we found that N-terminal deletion mutants, N (81-124) and N (101-124), failed to induce apoptosis. However, deletion of the N-terminal 40 amino acids (N (41-124)) did not affect the induction of apoptosis. On the other hand, C-terminal deletion mutant, N (1-71) still remained the pro-apoptotic function, but not N (1-20). Furthermore, truncation of minimal region comprising residue 41 to 71 (N (41-71)) was by itself sufficient to induce apoptosis.



Table I. Summary of cell death assay and the association with p75NTR for NADE and its mutants.

NADE Mutants	Cell Death		association with p75NTR
	p75NTR -	p75NTR +	
NADE (WT)	—	+	+
N (1-120)	—	+	+
N (1-112)	—	+	+
N (1-100)	+	+	—
N (1-71)	+	+	—
N (1-20)	—	—	—
N (41-124)	—	+	+
N (81-124)	—	—	+
N (101-124)	—	—	—
N (41-71)	+	+	—
N (L99A)	—	+	+
N (L94A,L97A,L99A)	—	—	—

In cell death assay, + represents that apoptotic cells are scored more than 30 % of 293T cells.  
 - represents that apoptotic cells are scored more than 10 % of 293T cells.

Interestingly, N (1-71), which lacks p75NTR-binding domain (residues 81 to 106), remained the pro-apoptotic function even in the absence of p75NTR, whereas expression of wild-type NADE without p75NTR did not induce apoptosis. 5 Similar results were obtained from N (41-71) and N (1-100), which failed to associate with p75NTR. In contrast, N (1-120), N (1-112) and N (41-124), which can associate with p75NTR, induced NGF-dependent apoptosis. Thus, C-terminal amino acid residues (72-112), designated 10 regulatory domain, are essential for NGF-dependent regulation of NADE-induced apoptosis.

*NADE NES is necessary for self-association, interaction with p75NTR and apoptosis.* In our previous report, we 15 observed that the C-terminal residues between amino acids 90-100 conform to functional NES motif, as indicated by its similarity to other known NESs such as HIV REV (Fig. 8A, (13)). We demonstrated that wild type NADE with intact NES localized in cytoplasm, but the GFP-NES mutant 20 (Leu-94 → Ala and Leu-97 → Ala) remains in nucleus. Since the NADE NES localizes in the regulatory domain, including p75NTR-binding domain at residues 81-106, NADE NES may play a role in the regulation of apoptosis induced by NADE. To analyze NADE NES function, we constructed 25 point mutants, N (L99A) and N (L94A, L97A, L99A), consisting of leucine to alanine conversions at residues 94 to 99 in NADE NES (Fig. 5B). First of all, expression and subcellular distribution of these mutants were visualized in 293T cells using the enhanced green 30 fluorescent protein GFP-NADE fusion proteins and monitored by fluorescence microscopy. GFP-N (L94A, L97A, L99A) can be observed both in the nucleus and in the cytoplasm (Fig. 8B), whereas wild-type NADE and N (L99A) localized in the cytoplasm. These results suggest that NES consensus

sequence of NADE is an important determinant of NADE subcellular localization.

We previously reported that in 293T, PC12 and nnr5 cells transfected with wild-type NADE, two bands (22 and 44 kDa) were detected in SDS-PAGE under reducing condition (13). Deletion mutant, N (1-71) failed to dimerize, although N (1-112) can be detected as a dimer form (data not shown). Next, we performed immunoblotting analysis of cell extracts prepared from 293T cells transiently transfected with N (L99A) and N (L94A, L97A, L99A) (Fig. 8C). The results showed that N (L94A, L97A, L99A) does not form dimer, although wild-type NADE and NADE L99A dimerized under the reducing condition, suggesting that NES is necessary for self-association of NADE and that the regulation of nuclear export of NADE may be linked to the association or dissociation of NADE monomer. Three of the key hydrophobic residues of NES, Leu 94, Leu 97 and Leu 99, resides in p75NTR binding domain of NADE, suggesting that the NES mutations may also be affecting the interaction of p75NTR with NADE. We performed *in vitro* binding assays using GST-fusion proteins to assess the interaction between these point mutants and p75NTR(ICD). As shown in Fig. 8D, the data showed that N (L94A, L97A, L99A) failed to associate with p75NTR(ICD), but not N (L99A). In addition, N (L94A, L97A) associated with p75NTR(ICD) much less efficiently than wild-type NADE (data not shown).

To study the effect of NES on apoptosis, we performed apoptosis assay using point mutants of NADE (Fig. 8E). The analysis showed that N (L94A, L97A, L99A) failed to induce apoptosis, whereas N (L99A) induced apoptosis. In addition, N (94A, L97A) also failed to induce apoptosis (data not shown). These results indicated that NADE NES motif is crucial for nuclear export, self-association and

interaction with p75NTR, and required for NGF-dependent p75NTR/NADE-induced apoptosis.

**Overexpression of NADE suppressed NF- $\kappa$ B activity.** The transcription factor NF- $\kappa$ B has been suggested to have both pro-apoptotic (18) and anti-apoptotic (19, 20) properties, depending on the cell type. To investigate the influence of NADE protein on NF- $\kappa$ B activity, we transfected with pcDNA3.1myc-His(-)A/mNADE (WT), pcDNA3p75NTR, pELAM-luciferase reporter, and the pRL-TK reporter constructs into 293T cells. Overexpression of p75NTR in 293T cells induced slight activation of NF- $\kappa$ B and NGF-treatment slightly enhanced the NF- $\kappa$ B activity in the presence of p75NTR (Fig. 9A). The basal activity of NF- $\kappa$ B in 293T cells was significantly reduced by overexpression of wild-type mNADE. Cotransfection of p75NTR with or without NGF-treatment did not have significant change on the NF- $\kappa$ B suppression induced by wild-type NADE.

We also performed luciferase reporter assay in PC12 and nnr5 cells. As shown in Fig. 9B, NGF activated NF- $\kappa$ B activity approximately 3-fold in PC12 cells. Overexpression of mNADE suppressed NF- $\kappa$ B activity both in the presence and absence of NGF. Similar results were also obtained in nnr5 cells, which lack trkA receptor (Fig. 9C).

**Mutational analysis of mNADE on NF- $\kappa$ B activity.** We further examined whether the inactive NADE mutants function as dominant-negative activators of NF- $\kappa$ B in 293T cells (Fig. 10). The expression of endogenous NADE was confirmed by anti- $\alpha$ -NADE antibody in 293T, PC12 and nnr5 cells (data not shown). Introduction of NADE mutants, N (1-60), N (1-120) and N (C121S) with an NF- $\kappa$ B luciferase reporter plasmid in 293T cells markedly leads to a 6 to 11-fold increase in luciferase activity. In addition, N (C121S)

slightly activates NF- $\kappa$ B activity in the absence of NGF in PC12 and nmr5 cells (data not shown).

To map the structure features of mNADE required for NF- $\kappa$ B suppression, we used a series of NADE deletion mutants. As shown in Table 2, deletions of the N-terminal amino acids, N (41-124), N (81-124) and N (101-124) remained ability of NF- $\kappa$ B suppression. N (1-120), N (1-100) and N (1-60) act as dominant negative activators, whereas N (1-90) suppressed NF- $\kappa$ B activity at levels comparable with that of the intact molecule. These results indicate that two distinct domains, residues 61-90 and 121-124, contribute to NF- $\kappa$ B suppression.

## DISCUSSION

The recent identification of distinct classes of receptor-associated signal transducers provided insights into how members of the TNF receptor superfamily initiate downstream signaling events (26). Death domain is essential for the transduction of death signals elicited by ligands in TNFR/Fas, belonged to superfamily with p75NTR. Several downstream targets of TNFR and Fas death domains (subtype 1) have been identified (27). They also contain death domain sequences, indicating a signaling mechanism triggered by the association of death domain-containing proteins, including self association of death domain such as MORT1/FADD (28). However, the functional role of p75NTR-death domain (subtype 2), including DAP kinase, ankyrin and NF- $\kappa$ B p100 and p105 is still unclear. Structural analysis by NMR suggested that death domain of p75NTR may indicate a potential site of interaction with downstream targets in ligand dependent manner (29). We have reported that NADE associates with death domain of p75NTR in NGF-dependent manner and induce apoptosis (13). To better understand the mechanism of NADE action, we have carried out mutational analysis to define sequences

necessary for NADE function. These studies revealed a NADE anatomy composed of modular domains mediating distinct activities.

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Table II. Summary of luciferase activity for NADE and its mutants.

	NADE Mutants	Relative Luciferase Activity
10	NADE (WT)	8
	N (1-120)	848
	N (1-100)	263
	N (1-90)	33
	N (1-60)	661
	N (1-20)	98
15	N (41-124)	43
	N (81-124)	31
	N (101-124)	29
	N (41-71)	22
	N (C121S)	1119
20	vector	100

The values shown represent luciferase activities relative to vector control.

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The deletion mapping was a first approach to attempt to elucidate regions necessary for NADE function. As shown in Fig. 5, 6A, 6B, 7, 11 and Table 1, the truncation of minimal region comprising residues 41 to 71, named pro-apoptotic domain, was by itself sufficient to induce apoptosis, but not depend on NGF. Since our previous studies demonstrated that p75NTR/NADE-induced apoptosis was NGF-dependent in 293T cells after the interaction of mNADE with p75NTR (13), NADE requires the NGF-dependent regulatory domain for this pro-apoptotic function. Further analysis of deletion mutants showed that mutants conserved with pro-apoptotic domain and p75NTR-binding domain induced apoptosis in NGF-dependent manner. On the other hand, mutants containing pro-apoptotic domain but not p75NTR-binding ability induced apoptosis even in the absence of p75NTR. These results indicate that pro-apoptotic domain is regulated by C-terminal region, named regulatory domain, which includes p75NTR-binding domain in response to NGF. We hypothesize that after the recruitment of NADE to p75NTR (ICD) in response to NGF (13), the conversion of NADE protein conformation triggers the exposure of the pro-apoptotic domain to unknown signal transducers which mediate their apoptosis to downstream. Future studies will be necessary to determine what are downstream target molecules for this pro-apoptotic domain.

To further analyze the structure features of regulatory domain necessary for ligand dependent function of NADE, we generated a series of NADE NES point mutants, because NES motif located in NADE regulatory domain (Fig. 8A, and 11). We constructed point mutants, N (L99A) and N (L94A, L97A, L99A), since analogous mutations in other NES-containing proteins have been reported to prevent nuclear export (24, 25). The results showed that N (L94A, L97A, L99A) decreased the efficiency of nuclear export, interaction with p75NTR and self-association, suggesting

that NADE NES motif is crucial for those functions. NES motif has been reported to require for self-association of p53 and may be linked to regulation of subcellular localization and p53 activity (30). NADE NES may be also  
5 important for the regulation of sublocalization, recruitment to p75NTR and induction of pro-apoptotic activity, although we have not observed the translocation of NADE to nucleus under physiological condition.

As shown in Fig. 9, 10 and 11, our data indicated  
10 that one of the unique features of NADE is its ability to suppress the NF- $\kappa$ B activity in 293T, PC12 and nnr5 cells. Several possibilities may explain the nature of NF- $\kappa$ B/Rel suppression by NADE. The mechanism of NF- $\kappa$ B/Rel activation has been well characterized (31). Subsequent  
15 phosphorylation of I $\kappa$ B subunits by IKK $\alpha$  and IKK $\beta$  leads to the specific ubiquitination and degradation of I $\kappa$ B by ubiquitin-proteasome pathways. Degradation of the I $\kappa$ Bs is necessary for NF- $\kappa$ B/Rel activation and translocation to the nucleus. Since NADE has ubiquitination sequences, it  
20 may modulate NF- $\kappa$ B/I B complex activity by competing for common ubiquitin substrates. The analysis of dominant negative form suggests that NADE may be a component of NF- $\kappa$ B signaling as a suppressor, resulting in reconstitution of steady-state levels of NF- $\kappa$ B. Recently, in several cell  
25 types and nervous system, NF- $\kappa$ B activation by NGF has been reported (8, 18-20, 23). The recruitment of NADE to p75NTR (ICD) in response to NGF may release the suppression effect and induce activation of NF- $\kappa$ B. It will be of interest to test whether NF- $\kappa$ B/Rel or I B are the  
30 potential protein substrates of NADE.



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**What is claimed is:**

1. An isolated nucleic acid molecule encoding a polypeptide capable of binding with a p75<sup>NTR</sup> receptor.
- 5 2. The isolated DNA molecule of claim 1.
3. The isolated cDNA molecule of claim 2.
- 10 4. The isolated RNA molecule of claim 1.
5. The isolated nucleic acid molecule of claims 1-4 encoding a neurotrophin associated cell death executor protein.
- 15 6. The isolated nucleic acid molecule of claims 1-4 which comprises a sequence of AATTG TCTAC GCATC CTTAT GGGGG AGCTG TCTAA C.
- 20 7. The isolated nucleic acid molecule of claim 5 which comprises a sequence of AATTG TCTAC GCATC CTTAT GGGGG AGCTG TCTAA C.
8. The isolated nucleic acid of claim 1 operatively linked to a promoter of RNA transcription.
- 25 9. A vector which comprises the isolated nucleic acid of claim 1, operatively linked to a promoter of RNA transcription.
- 30 10. The vector of claim 9, wherein the vector is plasmid.
11. The isolated nucleic acid molecule of claim 3, wherein the nucleic acid molecule encodes human or mouse polypeptide capable of binding p75<sup>NTR</sup> receptor.
- 35 12. The isolated nucleic acid molecule of claim 11, wherein the nucleic acid molecule encodes a polypeptide capable

of binding p75<sup>NTR</sup> receptor set forth in Figure 1G-1 (SEQ ID NO: \_\_).

13. The isolated nucleic acid molecule of claim 3, wherein  
5 the nucleic acid molecule encodes a polypeptide capable of binding p75<sup>NTR</sup> receptor.
14. The isolated nucleic acid molecule of claim 9 wherein  
10 the polypeptide capable of binding p75<sup>NTR</sup> receptor is mouse, rat or human protein.
15. The isolated nucleic acid of claim 3 which comprises the  
nucleic acid sequence set forth in Figure 1G-1 (SEQ ID  
15 NO: \_\_).
16. A host cell comprising the vector comprising the nucleic  
acid molecule of claim 1.
17. The host cell of claim 16, wherein the cell is selected  
20 from a group consisting of a bacterial cell, a plant cell, an insect cell, and a mammalian cell.
18. A method of producing a polypeptide capable of binding  
25 p75<sup>NTR</sup> receptor which comprises growing the host cells of claim 17 under suitable conditions permitting production of the polypeptide.
19. The method of claim 18 further comprising recovering the  
30 produced polypeptide.
20. An isolated nucleic acid molecule of at least 15  
contiguous nucleotides capable of specifically  
hybridizing with a unique sequence included within the  
sequence of the nucleic acid molecule of claim 1.  
35
21. The isolated nucleic acid of claim 20 which is a DNA molecule.

22. The isolated nucleic acid of claim 20 which is a RNA molecule.
23. An isolated nucleic acid molecule capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule which is complementary to the nucleic acid molecule of claim 1.
24. The isolated nucleic acid of claim 23 which is a DNA molecule.
25. The isolated nucleic acid of claim 23 which is a RNA molecule.
26. An antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to an mRNA molecule encoding a polypeptide capable of binding p75<sup>NTR</sup> receptor.
27. The antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to the cDNA molecule of claim 3.
28. The antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to the RNA molecule of claim 4.
29. A purified polypeptide capable of binding p75<sup>NTR</sup> receptor.
30. A purified polypeptide capable of binding p75<sup>NTR</sup> receptor encoded by the isolated nucleic acid of claim 1.
31. A purified unique polypeptide fragment of the polypeptide capable of binding p75<sup>NTR</sup> receptor of claim 30.
32. The polypeptide capable of binding p75<sup>NTR</sup> receptor of

claim 30 having substantially the same amino acid sequence as set forth in Figure 1G-1 (SEQ ID NO: \_\_).

- 5 33. The polypeptide capable of binding p75<sup>NTR</sup> receptor of claim 30 having the amino acid sequence as set forth in Figure 1G-1 (SEQ ID NO: \_\_).
- 10 34. The polypeptide capable of binding p75<sup>NTR</sup> receptor of claim 33 which is a vertebrate polypeptide capable of binding p75<sup>NTR</sup> receptor.
35. The polypeptide of claims 29-34 which comprises a neurotrophin associated cell death executor protein.
- 15 36. The polypeptide of claims 29-34 which comprises an amino acid sequence of NCLRILMGELSN.
- 20 37. The polypeptide of claim 35 which comprises an amino acid sequence of NCLRILMGELSN.
38. The vertebrate polypeptide capable of binding p75<sup>NTR</sup> receptor of claim 34 which is a mouse, rat, or human polypeptide capable of binding p75<sup>NTR</sup> receptor.
- 25 39. A monoclonal antibody directed to an epitope of a polypeptide capable of binding p75<sup>NTR</sup> receptor of claim 35.
- 30 40. A monoclonal antibody of claim 33 directed to a mouse, rat or human polypeptide capable of binding p75<sup>NTR</sup> receptor.
- 35 41. A polyclonal antibody directed to an epitope of the polypeptide capable of binding p75<sup>NTR</sup> receptor of claim 32.
42. A polyclonal antibody of claim 41 directed to a mouse, rat or human polypeptide capable of binding p75<sup>NTR</sup>

receptor.

43. A method of inducing apoptosis in cells which comprises  
expressing a polypeptide capable of binding p75<sup>NTR</sup>  
5 receptor in the cells.
44. A method of inducing apoptosis in a subject which  
comprises expressing a polypeptide capable of binding  
p75<sup>NTR</sup> receptor in a subject.  
10
45. The method of claim 44 where the subject is a rat, mouse  
or human.
46. A transgenic nonhuman mammal which comprises an isolated  
15 DNA molecule of claim 2.
47. The transgenic nonhuman mammal of claim 46, wherein the  
DNA encoding a polypeptide capable of binding p75<sup>NTR</sup>  
receptor is operatively linked to tissue specific  
20 regulatory elements.
48. A method of determining physiological effects of  
expressing varying levels of a polypeptide capable of  
binding p75<sup>NTR</sup> receptor in a transgenic nonhuman mammal  
25 which comprises producing a panel of transgenic non  
human mammal expressing a different amount of  
polypeptide capable of binding p75<sup>NTR</sup> receptor.
49. A method of producing a polypeptide capable of binding  
30 p75<sup>NTR</sup> receptor into a suitable vector which comprises:
- (a) inserting a nucleic acid molecule encoding the  
polypeptide capable of binding p75<sup>NTR</sup> receptor into  
a suitable vector;
  - 35 (b) introducing the resulting vector into a suitable  
host cell;
  - (c) selecting the introduced host cell for the  
expression of the polypeptide capable of binding



p75<sup>NTR</sup> receptor;

(d) culturing the selected cell to produce the polypeptide capable of binding p75<sup>NTR</sup> receptor; and

(e) recovering the polypeptide capable of binding p75<sup>NTR</sup> receptor produced.

5

50. A method of inducing apoptosis of cells in a subject comprising administering to the subject a purified polypeptide capable of binding p75<sup>NTR</sup> receptor in an amount effective to induce apoptosis.

10

51. The method of claim 50 wherein the subject is a mammal.

52. The method of claim 51, wherein the mammal is mouse, rat or human.

15

53. A pharmaceutical composition comprising a purified polypeptide capable of binding p75<sup>NTR</sup> receptor of either claim 32 or 33 and a pharmaceutically acceptable carrier.

20

54. A pharmaceutical composition comprising an effective amount of a purified polypeptide capable of binding p75<sup>NTR</sup> receptor of either claim 32 or 33 and a pharmaceutically acceptable carrier.

25

55. A method of identifying a compound capable of inhibiting binding between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> receptor comprising:

30

a) contacting the compound with the polypeptide capable of binding to p75<sup>NTR</sup> receptor under conditions permitting the binding of the polypeptide capable of binding to p75<sup>NTR</sup> receptor and p75<sup>NTR</sup> receptor to form a complex;

35

b) contacting the p75<sup>NTR</sup> receptor with the mixture from step a); and

c) measuring the amount of the formed complexes or

the unbound p75<sup>NTR</sup> receptor or the unbound polypeptide or any combination thereof.

56. A method of identifying a compound capable of inhibiting binding between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> receptor, where said binding forms a complex between p75<sup>NTR</sup> receptor and a polypeptide capable of binding p75<sup>NTR</sup> receptor, comprising:
- a) contacting the compound with the p75<sup>NTR</sup> receptor under conditions permitting the binding of the polypeptide capable of binding to p75<sup>NTR</sup> receptor and p75<sup>NTR</sup> receptor to form a complex;
  - b) contacting the p75<sup>NTR</sup> receptor with the mixture from step a); and
  - c) measuring the amount of the formed complexes or the unbound p75<sup>NTR</sup> receptor or the unbound polypeptide or any combination thereof.
57. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a neurotrophin associated cell death executor.
58. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a human HGR74 protein.
59. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a musnade3a sequence as defined on Figure 1H.
60. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a hunade3a1 sequence as defined on Figure 1H.
61. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a hunade3a2 sequence as defined on Figure 1H.

62. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a ratnad3a sequence as defined on Figure 1H.
- 5
63. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a ratnad3b sequence as defined on Figure 1H.
- 10 64. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a musnade3b sequence as defined on Figure 1H.
- 15 65. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a humnade1 sequence as defined on Figure 1H.
- 20 66. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a ratnade1 sequence as defined on Figure 1H.
- 25 67. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a musnade1 sequence as defined on Figure 1H.
68. The method of claims 55 or 56 wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a humnade2 sequence as defined on Figure 1H.
- 30 69. A method for identifying an apoptosis inducing compound comprising:
- 35 a) contacting a subject with an appropriate amount of the compound; and
- b) measuring the expression level of a polypeptide capable of binding p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene in the subject, an increase of the expression levels of a polypeptide capable of binding p75<sup>NTR</sup>

receptor gene and p75<sup>NTR</sup> gene indicating that the compound is an apoptosis inducing compound.

70. The method of claim 69 wherein the subject is a mammal.  
5
71. The method of claim 70, wherein the mammal is mouse, rat or human.
72. A method for identifying an apoptosis inducing compound  
10 comprising:
- a) contacting a cell with an appropriate amount of the compound; and
  - b) measuring the expression level of a polypeptide  
15 capable of binding a p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene in the cell, an increase of the expression levels of polypeptide capable of binding p75<sup>NTR</sup> receptor gene and p75<sup>NTR</sup> gene indicating that the compound is an apoptosis inducing compound.  
20
73. A method for screening cDNA libraries of a polypeptide capable of binding p75<sup>NTR</sup> receptor using a yeast two-hybrid system using a p75<sup>NTR</sup> intracellular domain as a target.  
25
74. The method of claim 73 where the cDNA library is mammalian.
75. The method of claim 74 where the mammalian cDNA library  
30 is derived from rat, mouse or human cDNA libraries.
76. The method of claim 73 where the p75<sup>NTR</sup> intracellular domain target is mammalian.
- 35 77. The method of claim 76 where the mammalian p75<sup>NTR</sup> intracellular domain target is a rat, mouse or human p75<sup>NTR</sup> intracellular domain target.

78. A method to induce caspase-2 and caspase-3 activity to cleave poly (ADP-ribose) polymerase and fragment nuclear DNA in a cell by co-expression of polypeptide capable of binding p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>.
- 5
79. A method to inhibit NF- $\kappa$ B activation in a cell with polypeptide capable of binding p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>.
- 10 80. A method to detect a neurodegenerative disease in a subject by detecting expression levels of a polypeptide capable of binding p75<sup>NTR</sup> receptor and p75<sup>NTR</sup>.
- 15
81. The method of claim 80 wherein the subject is a mammal.
82. The method of claim 81, wherein the mammal is mouse, rat or human.
- 20
83. A transgenic nonhuman mammal which comprises an isolated nucleic acid, encoding a human HGR74 protein, which is a DNA molecule.
- 25
84. The transgenic nonhuman mammal of claim 83 where the DNA encoding a human HGR74 protein is operatively linked to tissue specific regulatory elements.
- 30
85. A method of determining physiological effects of expressing varying levels of human HGR74 in a transgenic nonhuman mammal which comprises producing a panel of transgenic non human mammal expressing a different amount of human HGR74 protein.
- 35
86. A method of producing human HGR74 protein into a suitable vector which comprises:
- (a) inserting a nucleic acid molecule encoding a human HGR74 protein into a suitable vector;
  - (b) introducing the resulting vector into a suitable

- host cell;
- (c) selecting the introduced host cell for the expression of the human HGR74 protein;
  - (d) culturing the selected cell to produce the human HGR74 protein; and
  - (e) recovering the human HGR74 protein produced.
87. A method of inducing apoptosis of cells in a subject comprising administering to the subject the purified human HGR74 protein in an amount effective to induce apoptosis.
88. The method of claim 86 wherein the subject is a mammal.
89. The method of claim 87, wherein the mammal is mouse, rat or human.
90. A pharmaceutical composition comprising a purified human HGR74 protein and a pharmaceutically acceptable carrier.
91. A method for identifying an apoptosis inducing compound comprising:
- (a) contacting a subject with an appropriate amount of the compound; and
  - (b) measuring the expression level of human HGR74 protein gene and p75<sup>NTR</sup> gene in the subject, an increase of the expression levels of human HGR74 protein and p75<sup>NTR</sup> gene indicating that the compound is an apoptosis inducing compound.
92. The method of claim 91 wherein the subject is a mammal.
93. The method of claim 92, wherein the mammal is mouse, rat or human.
94. A method for identifying an apoptosis inducing compound

comprising:

- (a) contacting a cell with an appropriate amount of the compound; and
- 5 (b) measuring the expression level of human HGR74 protein gene and p75<sup>NTR</sup> gene in the cell, an increase of the expression levels of human HGR74 protein gene and p75<sup>NTR</sup> gene indicating that the compound is an apoptosis inducing compound.

10

95. A method for screening cDNA libraries human HGR74 protein using a yeast two-hybrid system using a p75<sup>NTR</sup> intracellular domain as a target.

15 96. The method of claim 95 where the cDNA library is mammalian.

97. The method of claim 96 where the mammalian cDNA library is derived from rat, mouse or human cDNA libraries.

20

98. The method of claim 95 where the p75<sup>NTR</sup> intracellular domain target is mammalian.

25 99. The method of claim 98 where the mammalian p75<sup>NTR</sup> intracellular domain target is a rat, mouse or human p75<sup>NTR</sup> intracellular domain target.

30 100. A method to induce caspase-2 and caspase-3 activity to cleave poly (ADP-ribose) polymerase and fragment nuclear DNA in a cell by co-expression of human HGR74 protein and p75<sup>NTR</sup>.

101. A method to inhibit NF- $\kappa$ B activation in a cell with human HGR74 protein and p75<sup>NTR</sup>.

35

102. A method to detect a neurodegenerative disease in a subject by detecting expression levels of human HGR74 protein and p75<sup>NTR</sup>.

103. The method of claim 102 wherein the subject is a mammal.

104. The method of claim 103, wherein the mammal is human.

5

105. A method of identifying a compound, which is an apoptosis inhibitor, said compound is capable of inhibiting specific binding between polypeptide capable of binding p75<sup>NTR</sup> receptor and p75<sup>NTR</sup> receptor, so as to prevent apoptosis which comprises:

10

- (a) contacting the polypeptide capable of binding p75<sup>NTR</sup> receptor with a plurality of compounds under conditions permitting binding between a known compound previously shown to be able to displace a polypeptide capable of binding p75<sup>NTR</sup> receptor and the p75<sup>NTR</sup> receptor and the bound p75<sup>NTR</sup> receptor to form a complex; and
- (b) detecting the displaced polypeptide capable of binding p75<sup>NTR</sup> receptor or the complex formed in step (a), wherein the displacement indicates that the compound is capable of inhibiting specific binding between the polypeptide capable of binding p75<sup>NTR</sup> receptor and the p75<sup>NTR</sup> receptor.

15

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106. The method of claim 105, wherein the inhibition of specific binding between the polypeptide capable of binding p75<sup>NTR</sup> receptor and the p75<sup>NTR</sup> receptor affects the transcription activity of a reporter gene.

30

107. The method of claim 106, where in step (b) the displaced polypeptide capable of binding p75<sup>NTR</sup> receptor or the complex is detected by comparing the transcription activity of a reporter gene before and after the contacting with the compound in step (a), where a change of the activity indicates that the specific binding between the polypeptide capable of binding p75<sup>NTR</sup>

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receptor and the p75<sup>NTR</sup> receptor is inhibited and the polypeptide capable of binding p75<sup>NTR</sup> receptor is displaced.

5 108. The method of claim 105, wherein the p75<sup>NTR</sup> receptor is bound to a solid support.

109. The method of claim 105, wherein the compound is bound to a solid support.

10

110. The method of claim 105, wherein the compound comprises an antibody, an inorganic compound, an organic compound, a peptide, a peptidomimetic compound, a polypeptide or a protein.

15

111. The method of claim 105 wherein the contacting of step (a) is in vitro.

112. The method of claim 105, wherein the contacting of step  
20 (a) is in vivo.

113. The method of claim 112, wherein the contacting of step (a) is in a yeast cell.

25 114. The method of claim 112, wherein the contacting or step (a) is in a mammalian cell.

115. The method of claim 105, wherein the polypeptide capable of binding p75<sup>NTR</sup> receptor is a cell surface receptor.

30

116. The method of claim 112, wherein the cell-surface receptor is the p75 receptor.

117. The method of claim 105 where in the polypeptide capable  
35 of binding p75<sup>NTR</sup> receptor is a neurotrophin associated cell death exectuor.

118. The method of claim 105 where in the polypeptide capable

101

of binding p75<sup>NTR</sup> receptor is a human HGR74 protein.

119. The method of claim 105 wherein the polypeptide capable  
of binding p75<sup>NTR</sup> receptor is a neurotrophin associated  
5 cell death executor.

120. The method of claim 105 wherein the polypeptide capable  
of binding p75<sup>NTR</sup> receptor is a human HGR74 protein.

10 121. The method of claim 105 wherein the polypeptide capable  
of binding p75<sup>NTR</sup> receptor is a musnade3a sequence as  
defined on Figure 1H.

122. The method of claim 105 wherein the polypeptide capable  
15 of binding p75<sup>NTR</sup> receptor is a hunade3a1 sequence as  
defined on Figure 1H.

123. The method of claim 105 wherein the polypeptide capable  
of binding p75<sup>NTR</sup> receptor is a hunade3a2 sequence as  
20 defined on Figure 1H.

124. The method of claim 105 wherein the polypeptide capable  
of binding p75<sup>NTR</sup> receptor is a ratnad3a sequence as  
defined on Figure 1H.

25 125. The method of claim 105 wherein the polypeptide capable  
of binding p75<sup>NTR</sup> receptor is a ratnad3b sequence as  
defined on Figure 1H.

30 126. The method of claim 105 wherein the polypeptide capable  
of binding p75<sup>NTR</sup> receptor is a musnade3b sequence as  
defined on Figure 1H.

127. The method of claim 105 wherein the polypeptide capable  
35 of binding p75<sup>NTR</sup> receptor is a humnadel sequence as  
defined on Figure 1H.

128. The method of claim 105 wherein the polypeptide capable

of binding p75<sup>NTR</sup> receptor is a ratnadel sequence as defined on Figure 1H.

129. The method of claim 105 wherein the polypeptide capable  
5 of binding p75<sup>NTR</sup> receptor is a musnadel sequence as defined on Figure 1H.
130. The method of claim 105 wherein the polypeptide capable  
10 of binding p75<sup>NTR</sup> receptor is a humnade2 sequence as defined on Figure 1H.
131. An isolated nucleic acid molecule encoding a deletion  
mutant of a wild type polypeptide capable of binding  
with a p75<sup>NTR</sup> receptor, designated neurotrophin  
15 associated cell death executor protein (NADE), wherein the N-terminal 1-40 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(41-124), and the NADE N(41-124) induces apoptosis in the presence of p75<sup>NTR</sup>.  
20
132. An isolated nucleic acid molecule encoding a deletion  
mutant of a wild type polypeptide capable of binding  
with a p75<sup>NTR</sup> receptor, designated neurotrophin  
associated cell death executor protein (NADE), wherein  
25 the C-terminal 72-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(1-71), and the NADE N(1-71) induces apoptosis in the presence of p75<sup>NTR</sup> and in the absence of p75<sup>NTR</sup>.  
30
133. An isolated nucleic acid molecule encoding a deletion  
mutant of a wild type polypeptide capable of binding  
with a p75<sup>NTR</sup> receptor, designated neurotrophin  
associated cell death executor protein (NADE), wherein  
35 the N-terminal 1-40 amino acids and the C-terminal 72-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(41-71), and the NADE N(41-71) induces apoptosis in the

presence of p75<sup>NTR</sup> and in the absence of p75<sup>NTR</sup>.

134. An isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the C-terminal 121-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(1-120) and the NADE N(1-120) induces apoptosis in the presence of p75<sup>NTR</sup>.
135. An isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the C-terminal 113-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(1-112) and the NADE N(1-112) induces apoptosis in the presence of p75<sup>NTR</sup>.
136. An isolated nucleic acid molecule encoding a deletion mutant of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the C-terminal 101-124 amino acids of wild type NADE polypeptide have been deleted and the deletion mutant is designated NADE N(1-100) and the NADE N(1-100) induces apoptosis in the presence of p75<sup>NTR</sup> and in the absence of p75<sup>NTR</sup>.
137. An isolated nucleic acid molecule encoding a mutation of a wild type polypeptide capable of binding with a p75<sup>NTR</sup> receptor, designated neurotrophin associated cell death executor protein (NADE), wherein the point mutation results in Ala at amino acid position 99 for Leu at amino acid position of wild type NADE polypeptide, wherein the substitution mutant polypeptide is designated NADE N(L99A) and the NADE N(L99A) induces

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apoptosis in the presence of p75<sup>NTR</sup>.

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1/29

Mouse	NADE	1	MANVHQENEMEQLONGEEDRPVGGEGHQHPAGNNNNNNHNNHNNHRR	.....
Human	NADE	1	MANIHQENEMEQLONGEEDRPVGGEGHQHPAGN-----RR	
Mouse	NADE	51	GQARRLAPNFRWAI PNQMN DGLGGDDMMFMFMEEMREIRRKLRQLQR	* * *
Human	NADE	38	GQARRLAPNFRWAI PNQIN DGMGGDDMEIFMEEMREIRRKLRQLQR	
Mouse	NADE	101	NCLRLMGE LSNHHDDHDEFCLMP	124
Human	NADE	88	NCLRLMGE LSNHHDDHDEFCLMP	111

# Figure 1A

2/29

cZyxin	319-331	<u>L</u>	T	M	K	E	V	E	E	L	E	L	L	T
MAPKK	32- 44	A	<u>L</u>	Q	K	K	L	E	E	L	E	L	D	E
PKI- $\alpha$	37- 46		<u>L</u>	A	<u>L</u>	K	L	A	G	L	D	I		
TFIIIA	330-338			<u>L</u>	P	V	L	E	N	L	T	L		
RevHIV-1	73- 81			<u>L</u>	P	P	L	E	R	L	T	L		
RanBP1	178-189		K	<u>V</u>	A	E	K	L	E	A	L	S	V	R
FMRP	425-437	E	V	D	Q	<u>L</u>	R	L	E	R	L	Q	I	D
Gle1	351-356				<u>L</u>	P	L	G	K	L	T	L		
RexHTLV-1	81- 94	A	<u>L</u>	S	A	Q	L	Y	S	S	L	S	L	D S
human NADE	65- 77	R	E	<u>I</u>	R	R	K	L	R	E	L	Q	L	R
mouse NADE	88-100	R	E	<u>I</u>	R	R	K	L	R	E	L	Q	L	R

Figure 1B

3/29

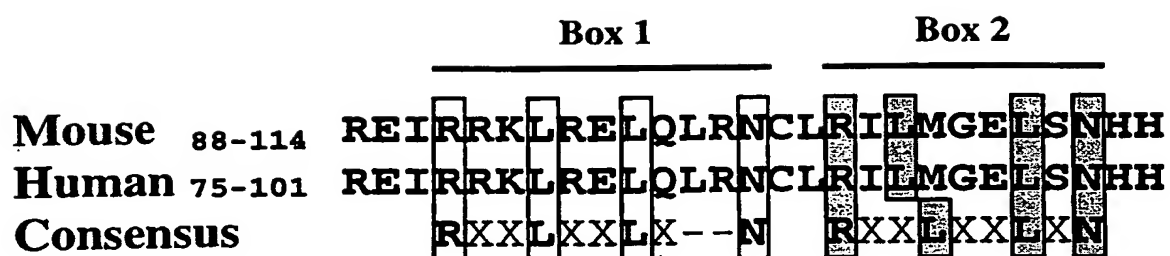
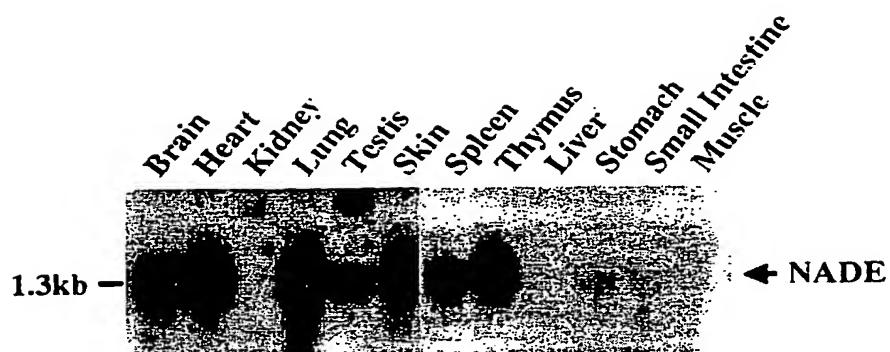


Figure 1 C



4/29

**Figure 1D**

5/29

Figure 1E

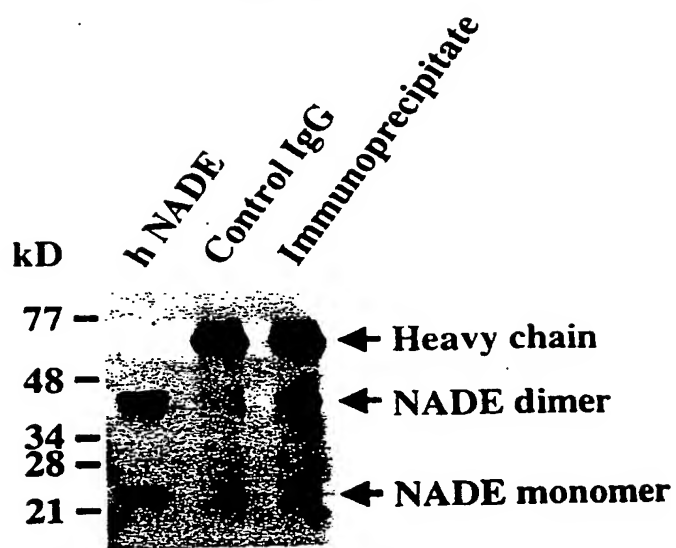
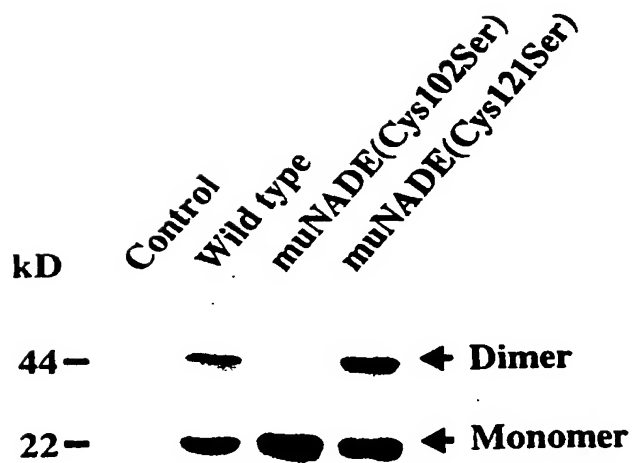


Figure 1F



6/29

Figure 1G-1

**Mouse**

```

1  acgagcgctctggccagcagctcggagctcctctgcgcgsggggggctggcagcggggcccg  60
61  aggcgagcgggacagattgactggaagccgagagtcaggcggcagcgggaattgacagg  120
121  aggactacgccgcaagggataggcccagaatagcaaccaggaaacaaaatctcatcatgg  180
181  ccaatgtccaccaggaaaacgaagagctggagcagccctgcagaatggacaggaagacc  240
241  gccctgtgggaggaggtgagggccaccagcctgctgcaaacaacaacaacaaccaca  300
301  accataaccacaaccaccaccgaagaggccaggtcgcgcgacttgccccctaacttccgat  360
361  gggccattcccaacaggcagatgaatgacgggttgggtggagatggagatgatatggaaa  420
421  tgttcattggaggagatgagagagatccggagaaagcttagggagctacagctgagaaatt  480
481  gtctacgcattccttatgggggagctgtcttaaccaccacgatcaccatgatgaattctgcc  540
541  ctatgccttgacttcgggtcattcccccttgagatccatactgtgactcccgctgtagccc  600
601  ttttcctcgcatttttctgacatgcccttaatgaccgcgttctgtggtgagccttctgttat  660
661  tcccatgccatgtgccaggtggggcctgtgttgccagtga

```

**Human**

```

1  accccatccccactcctataccggctcctccattttgggtgcctgcaaagctctgggaaag  60
60  aatcccgggaaacgaaaaatgggtgggttgggggaaggaggtaaggggagaaagctgga  120
121  gggaggggctttaattggaggccccgttagaggacgcgcggaacttctaagggtgggaaaaa  180
181  acgaaattaaaaaatcctttgatatacagggtcttgaatcctgctggtcagagcaccaagc  240
241  attcagtctctctccttgcctttgtcttacttgtgttcaaagaaaaacaaccagaaaaaa  300
301  aaaatctcatcatggcacaatattcaccaggaaaacgaagagatggagcagcctatgcaga  360
361  atggagaggaagaccgccccctgggaggagggtgaaggccaccagcctgcaggaaatcgac  420
421  ggggacaggctcgcgcgacttgccccctaatttctgatggggccatacccaataggcagatca  480
481  atgatgggatgggtggagatggagatgatatggaaatattcatggaggagatgagagaaa  540
541  tcagaagaaaacttagggagctgcagctgaggaattgtctgcgtatccttatggggggagc  600
601  ccttaataccatgaccatcatgatgaatttgccttatgccttgactcctgccattta  660
661  ccatgagattaatactgtgattcccgctgttttcttttcttcttgcattttccctaatatgc  720
721  ctttactgatecgtttgctgtgaacctatgttatttccatgtgtcaagtgggtcttctgt  780
781  tggcagcttctatttgaagattgccttgcactcagtgtaagtttctgtcagcagtagt  840
841  ttcaccatttgcatggaaaaatttaagctaataaagcaatttaaaaagc

```

Figure 1G-2

**7/29**

[illegible]

Page 2.1

	91	105	106	120	121	135	136	150	151	165	166	180
1	musnade3a	GDV	QRLME---	KLRE	QQLSHSLRAVSTDP	PP-	HHDHDEFC	CLMP				
2	hunade3a1	EEV	QRLME---	KLRE	QQLSHSLRAVSTDP	PP	HHDHDEFC	CLMP				
3	hunade3a2	EEV	QRLME---	KLRE	QQLSHSLRAVSTDP	PP	HHDHDEFC	CLMP				
4	ratnad3a	EDM	QRLME---	KLRE	QQLSHSLRAVSTDP	PP	HHDHDEFC	CLMP				
5	ratnad3b	RFV	QVQWEAKR	KSKE	QQVRPYTRFRTP	PEPD	NHYD----	FCLIP				
6	musnade3b	RFV	QVQSTEVR	KRTTTE	QQVRPYTRFRTP	PEPD	NHYD----	FCLIP				
7	humade1	MFMEEMRE	TRR	KLRE	LQLRNCILRI	LMGELS	NHDDHDEFC	CLMP				
8	ratnade1	MFMEEMRE	TRR	KLRE	LQLRNCILRI	LMGELS	NHDDHDEFC	CLMP				
9	musnade1	MFMEEMRE	TRR	KLRE	LQLRNCILRI	LMGELS	NHDDHDEFC	CLMP				
10	humade2	RFMEEMRE	LFR	KIRE	LQLRYSILRI	LIGDPP	HHDHDEFC	CLMP				

## Figure 1H

8/29

Figure 2A

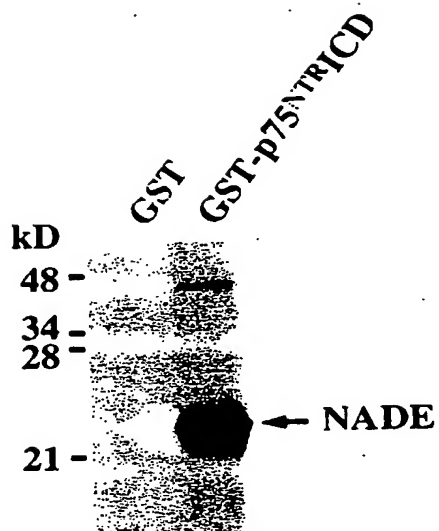
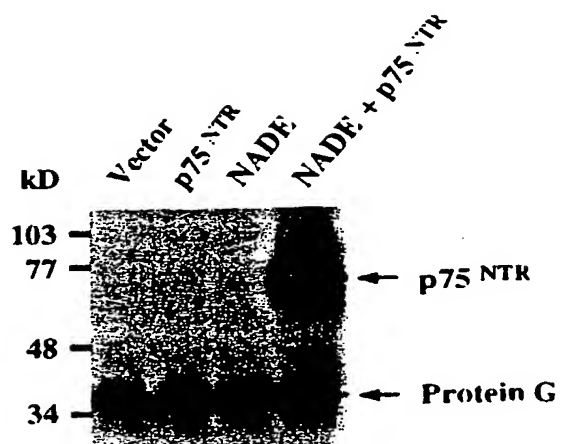


Figure 2B



9/29

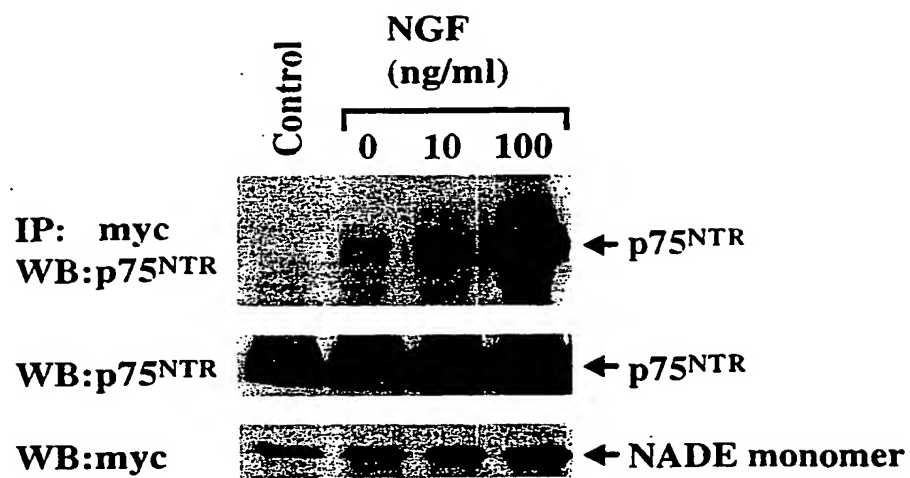
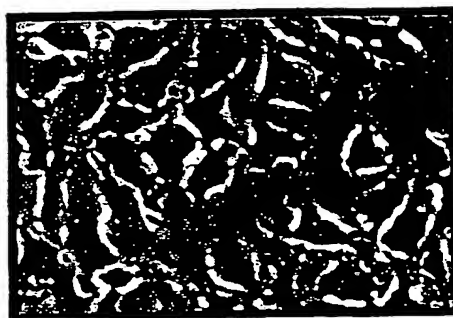


Figure 2C

10/29



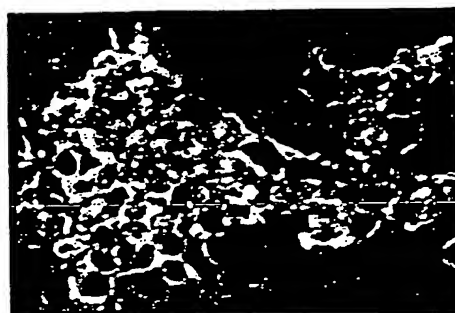
Control



NADE



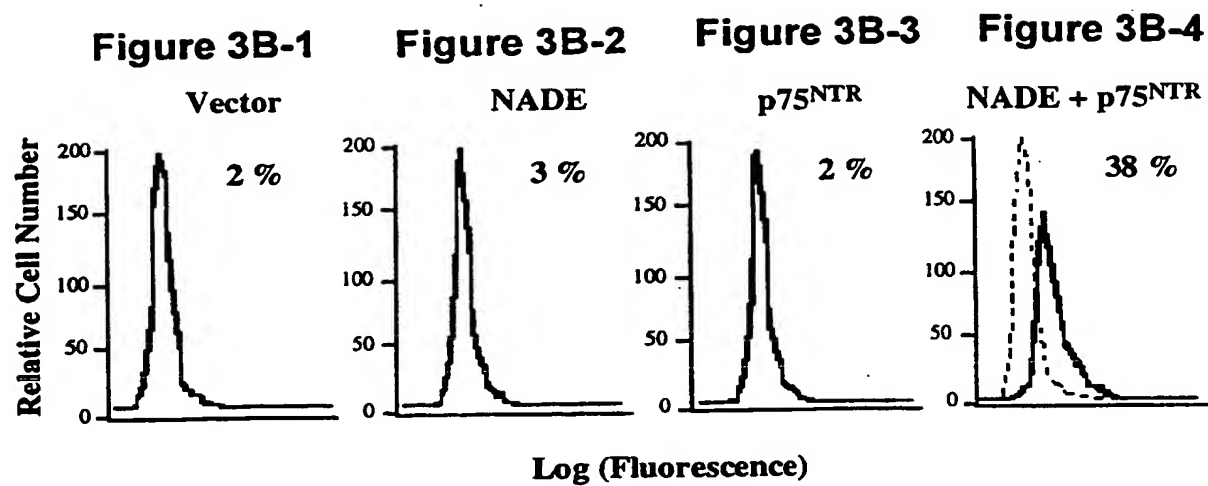
p75<sup>NTR</sup>



NADE + p75<sup>NTR</sup>

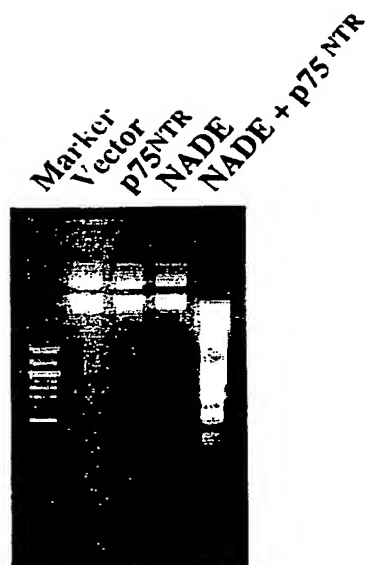
Figure 3 A

11/29



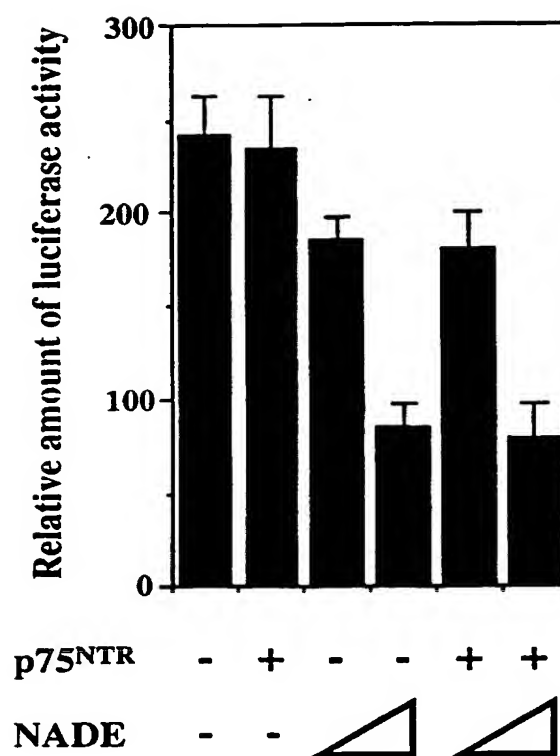


12/29



**Figure 3C**

13/29

**Figure 3D**

14/29

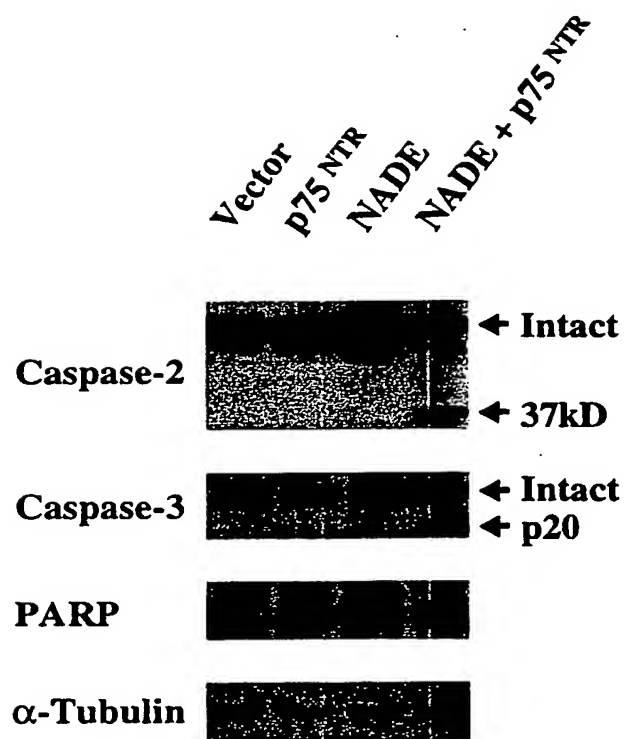
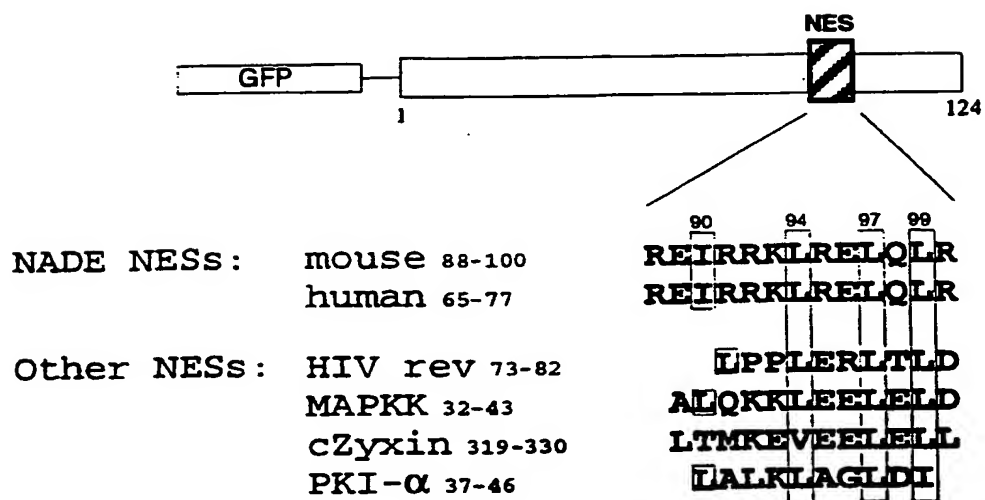


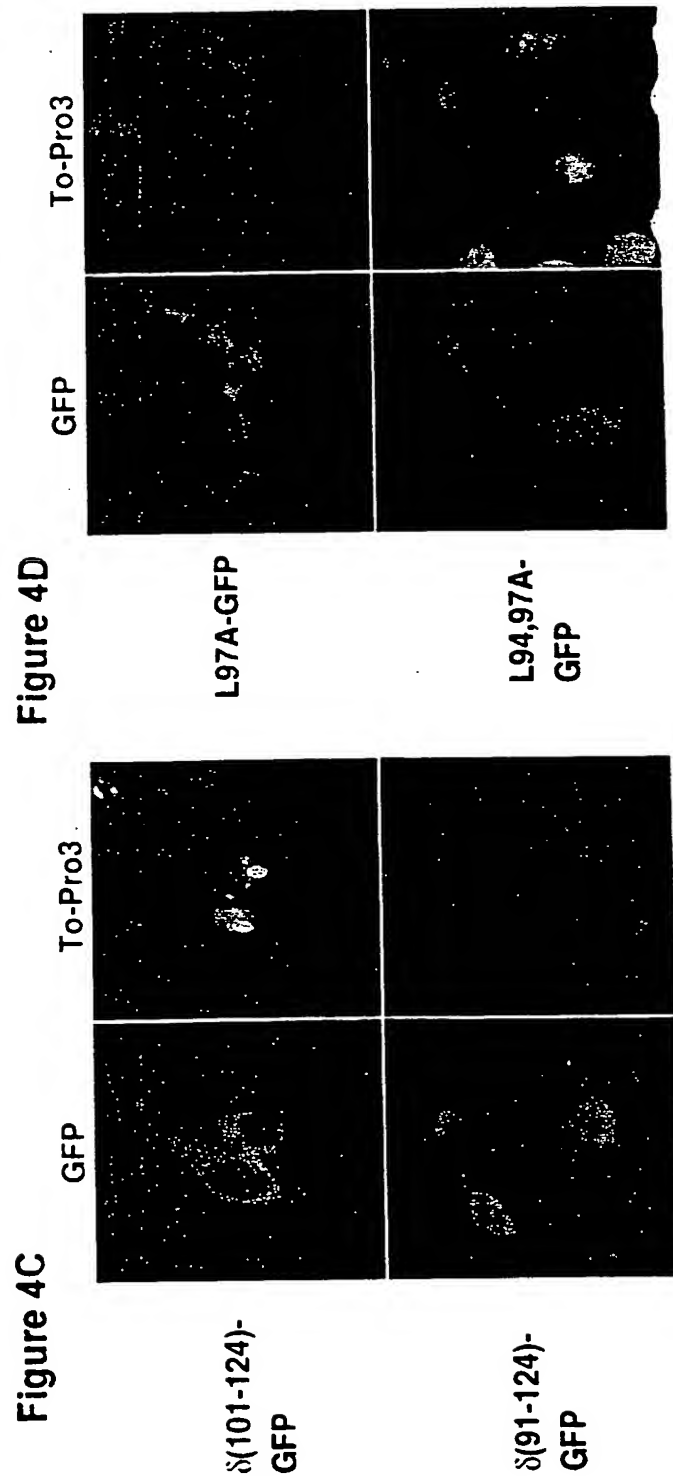
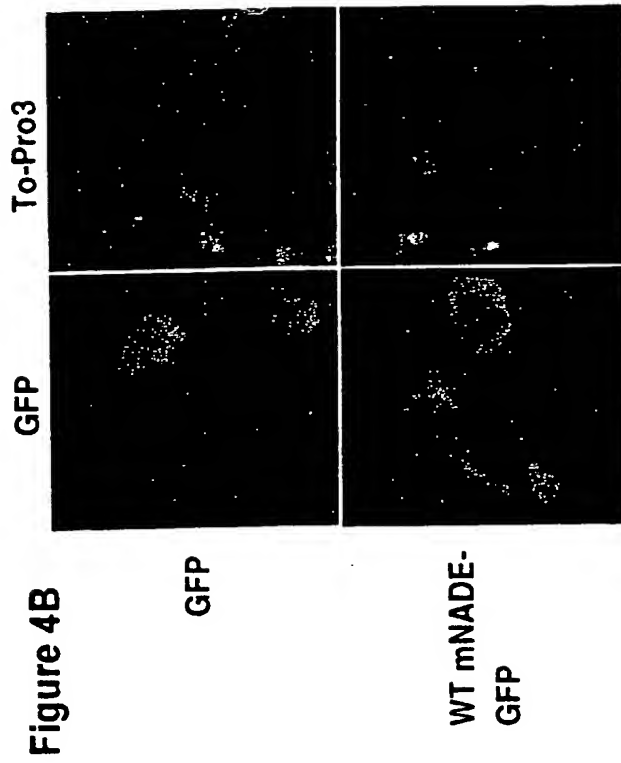
Figure 3E

15/29

**Figure 4A**  
**WT mouse NADE-GFP**



16/29



17/29

FIGURE 5A

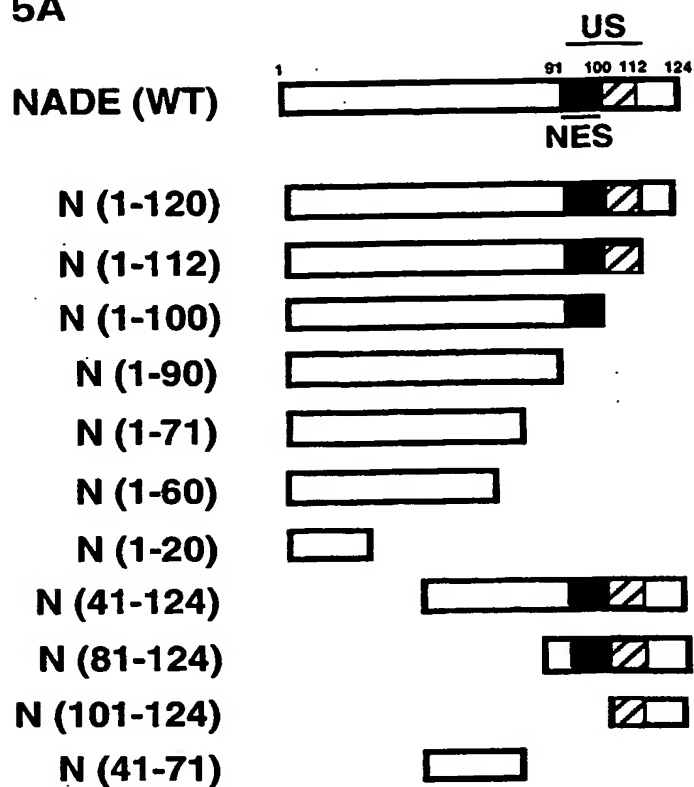
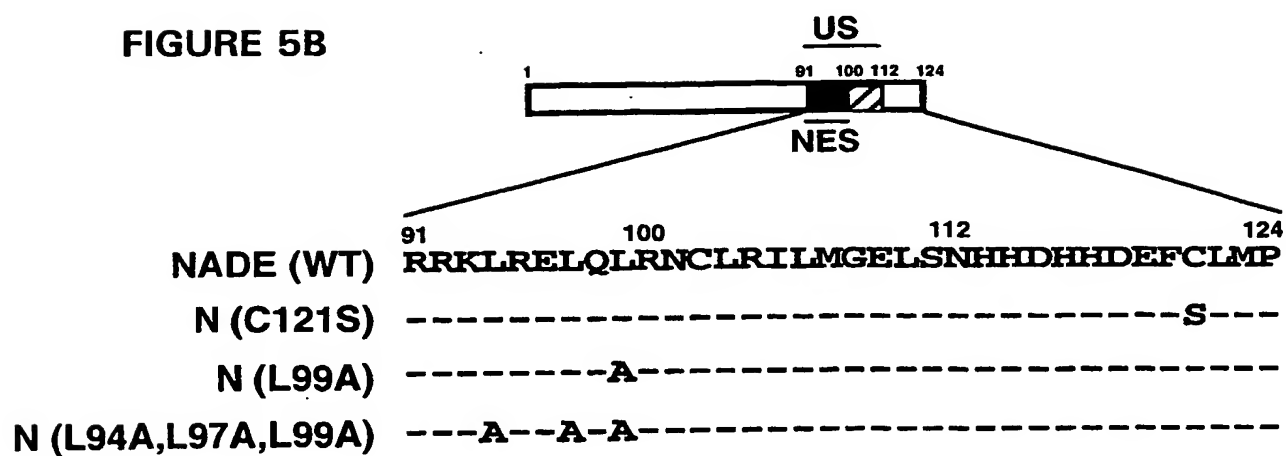


FIGURE 5B



18/29

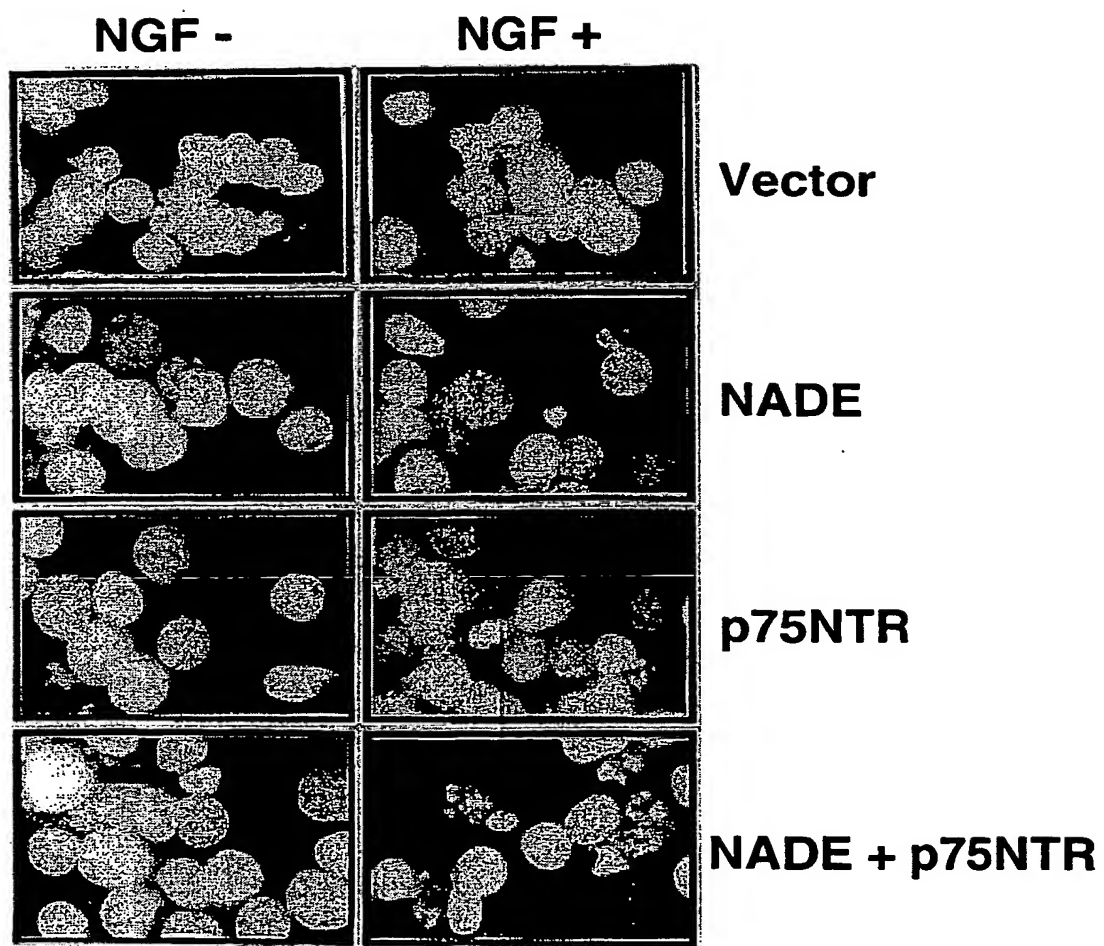


FIGURE 6A

19/29

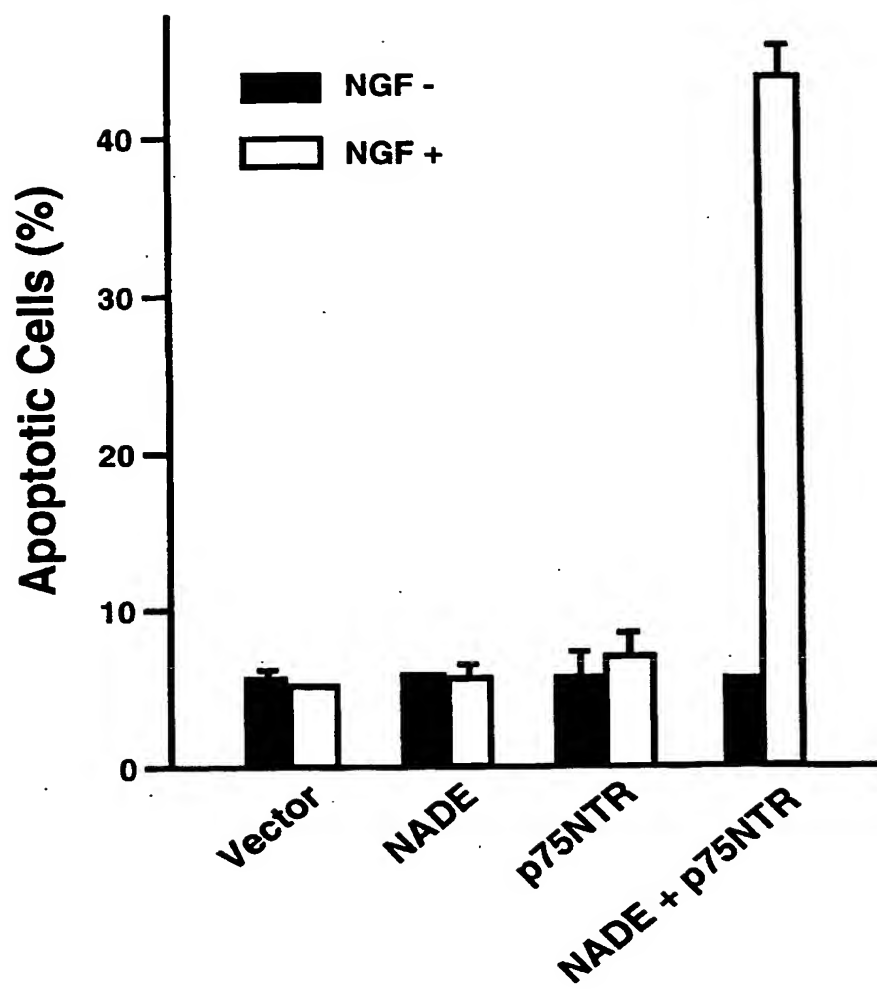


FIGURE 6B



20/29

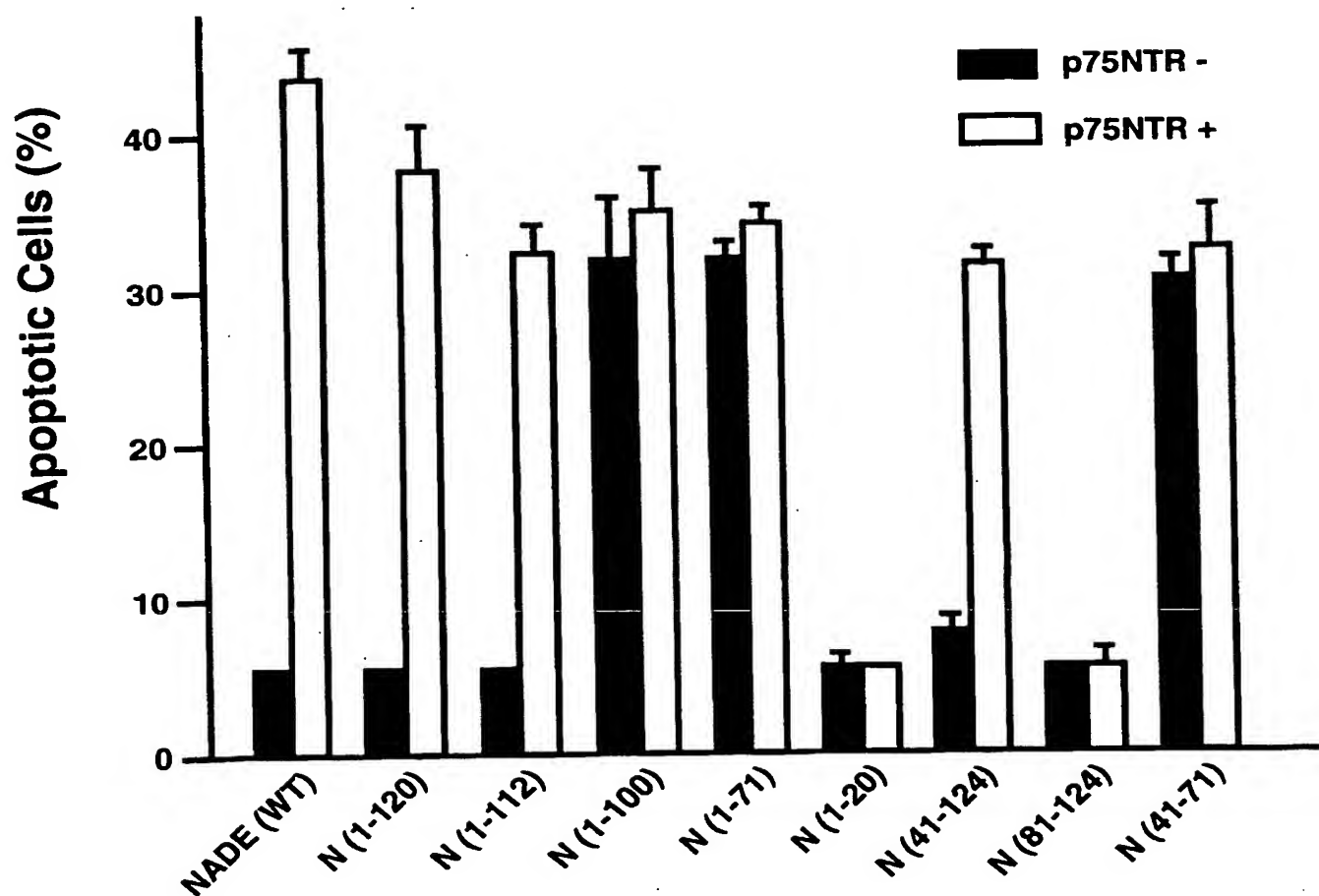


FIGURE 7

21/29

<b>NADE NESs:</b>	<b>mouse</b> 88-100	<b>RE</b>	<b>I</b>	<b>RR</b>	<b>KL</b>	<b>RE</b>	<b>LQ</b>	<b>LR</b>
	<b>rat</b> 84-96	<b>RE</b>	<b>I</b>	<b>RR</b>	<b>KL</b>	<b>RE</b>	<b>LQ</b>	<b>LR</b>
	<b>human</b> 65-77	<b>RE</b>	<b>I</b>	<b>RR</b>	<b>KL</b>	<b>RE</b>	<b>LQ</b>	<b>LR</b>
<b>Other NESs:</b>	<b>PKI</b> 37-45					<b>L</b>	<b>A</b>	<b>L</b>
	<b>HIV rev</b> 73-82					<b>L</b>	<b>P</b>	<b>L</b>
	<b>MDM2</b> 197-206					<b>L</b>	<b>S</b>	<b>F</b>
	<b>MAPKK</b> 32-43					<b>A</b>	<b>L</b>	<b>L</b>

FIGURE 8A

22/29

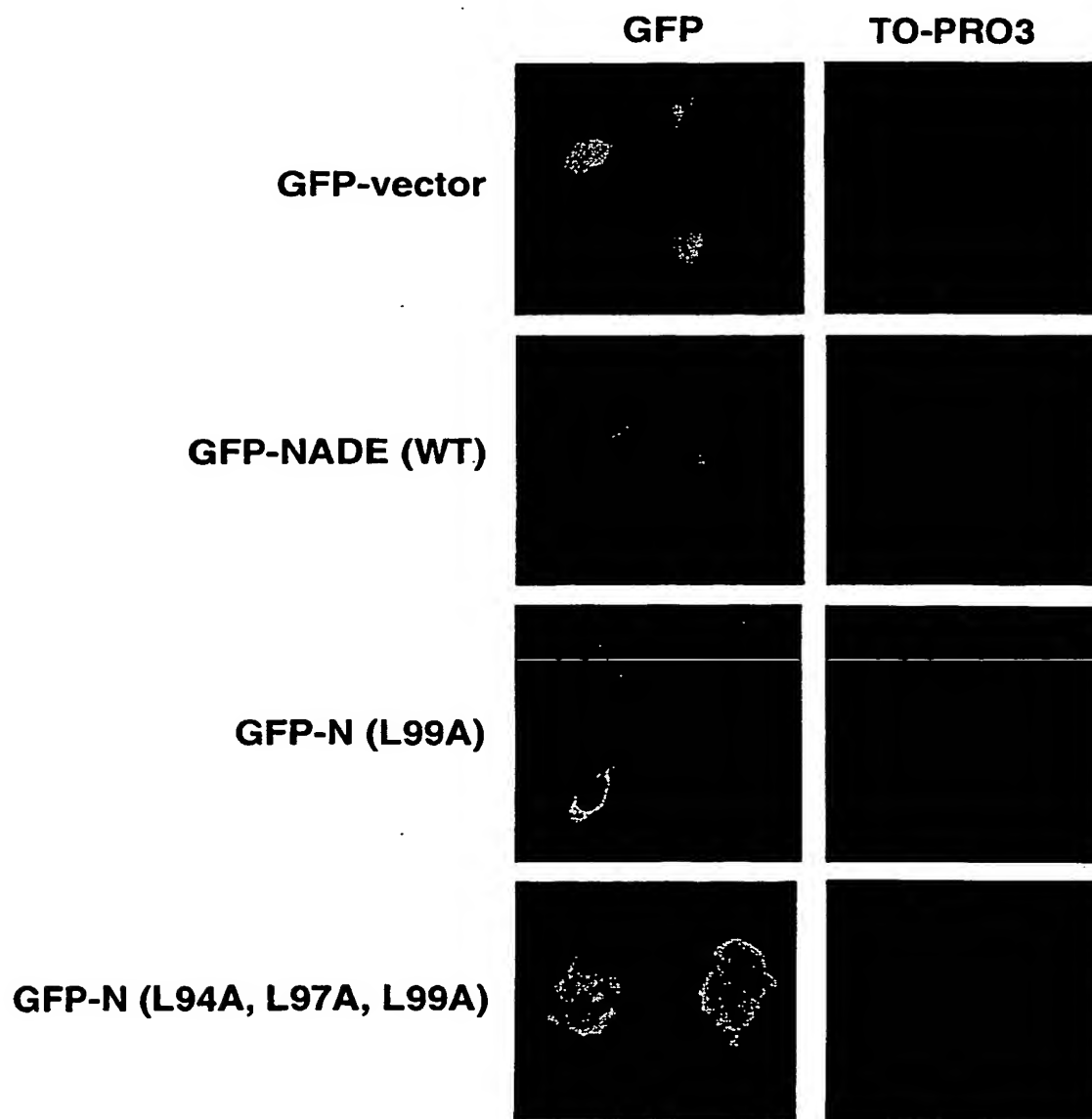


FIGURE 8B

23/29

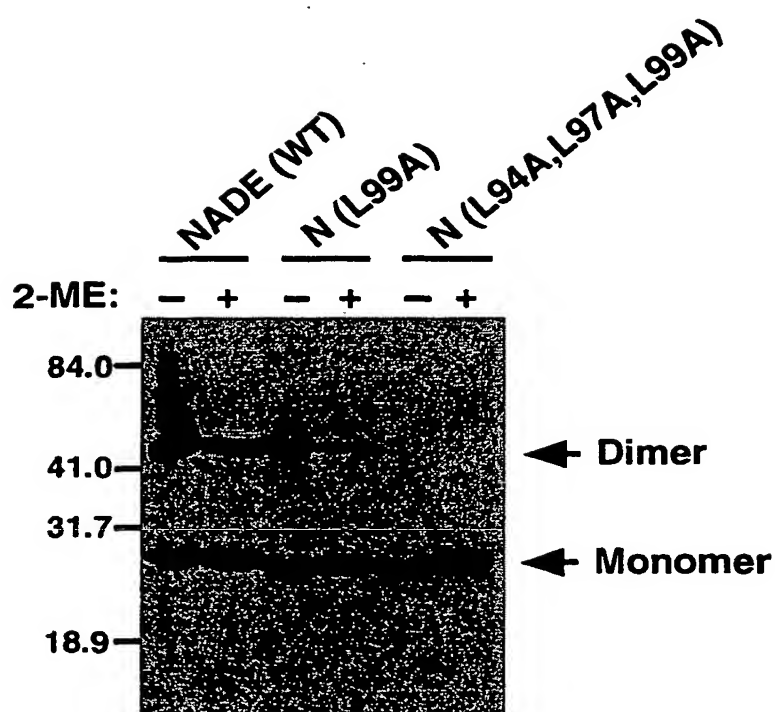


FIGURE 8C

24/29

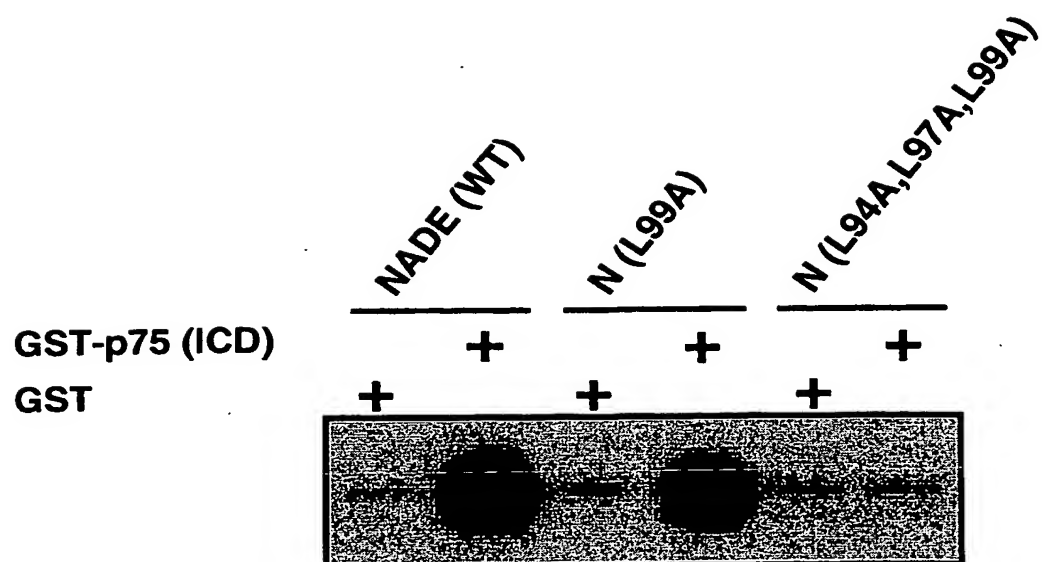


FIGURE 8D

25/29

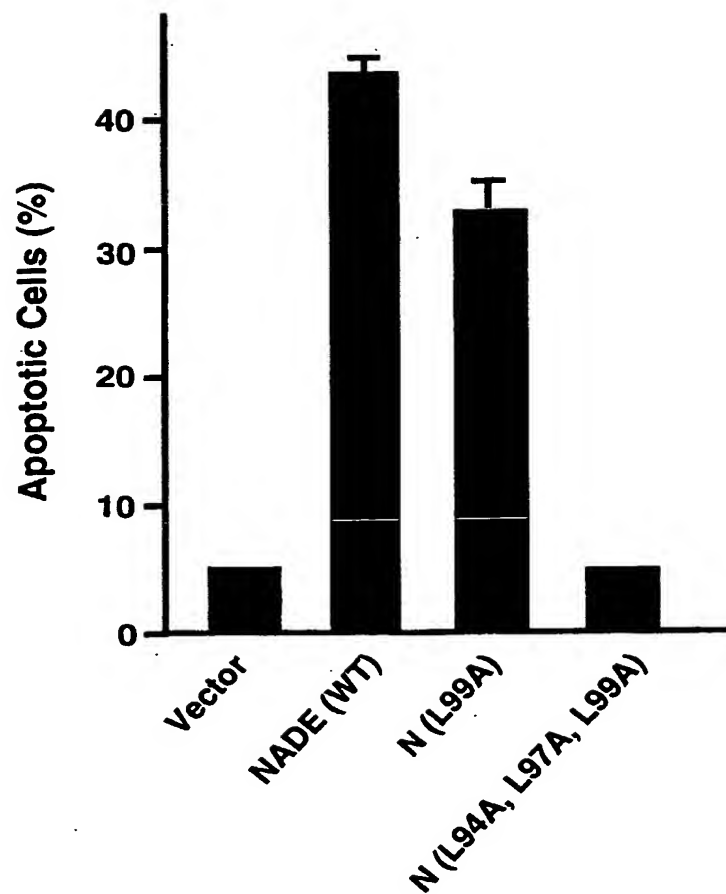


FIGURE 8E

26/29

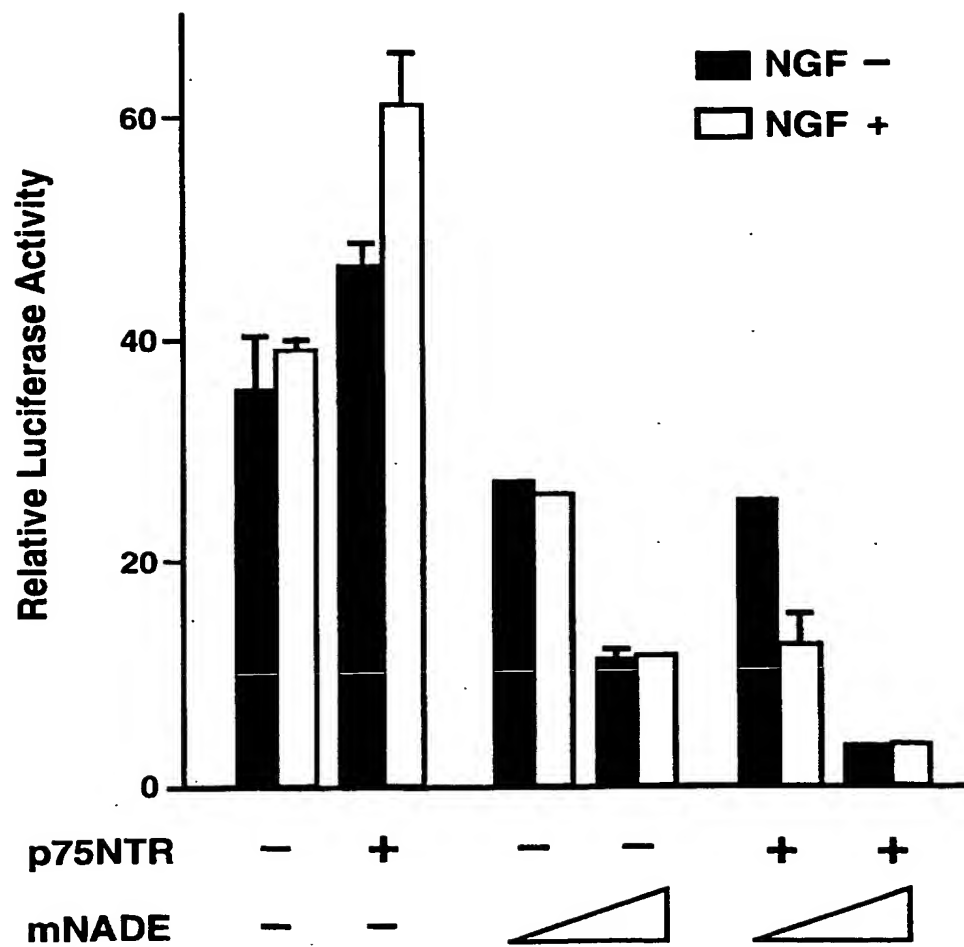
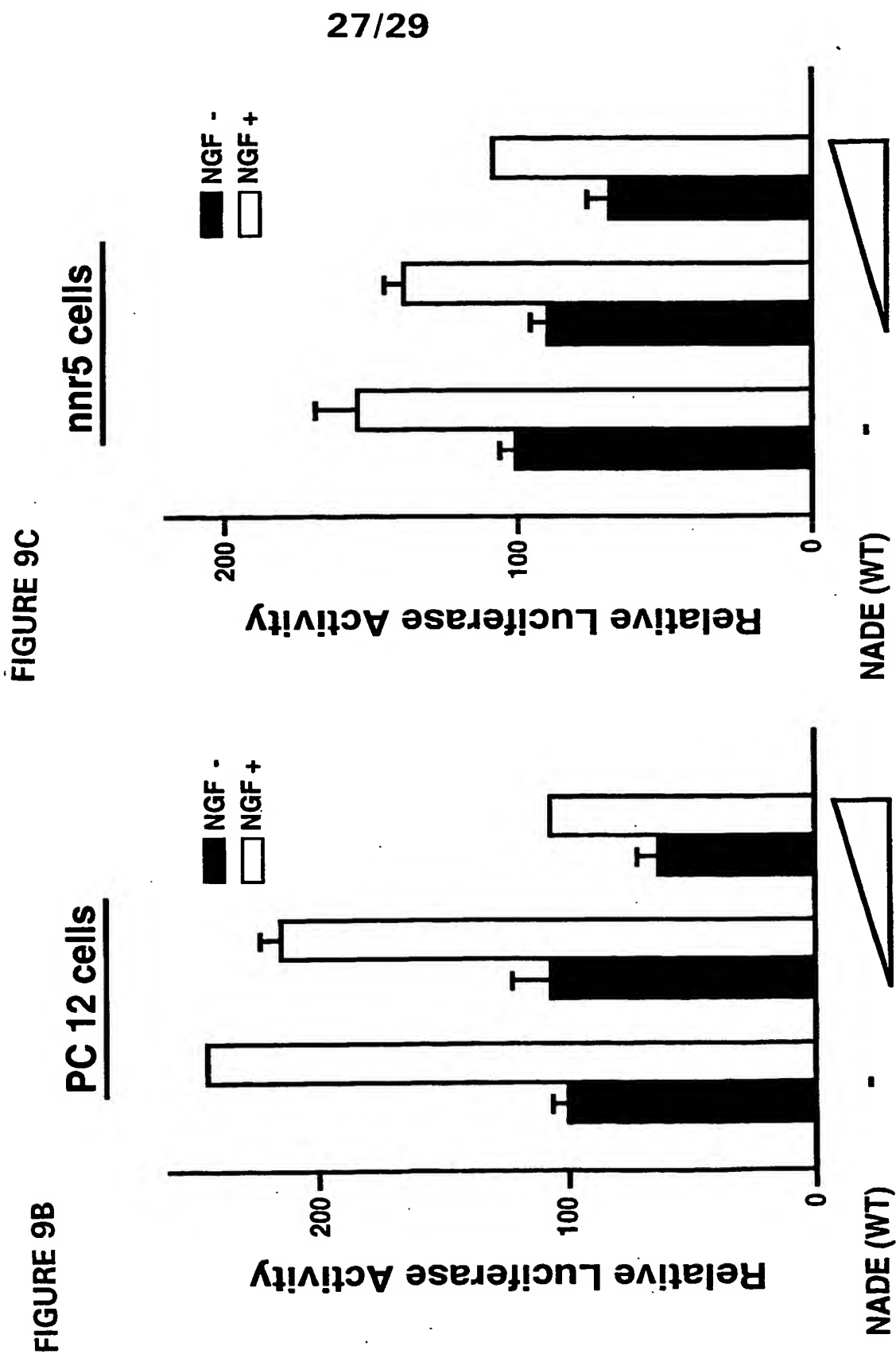


FIGURE 9A





28/29

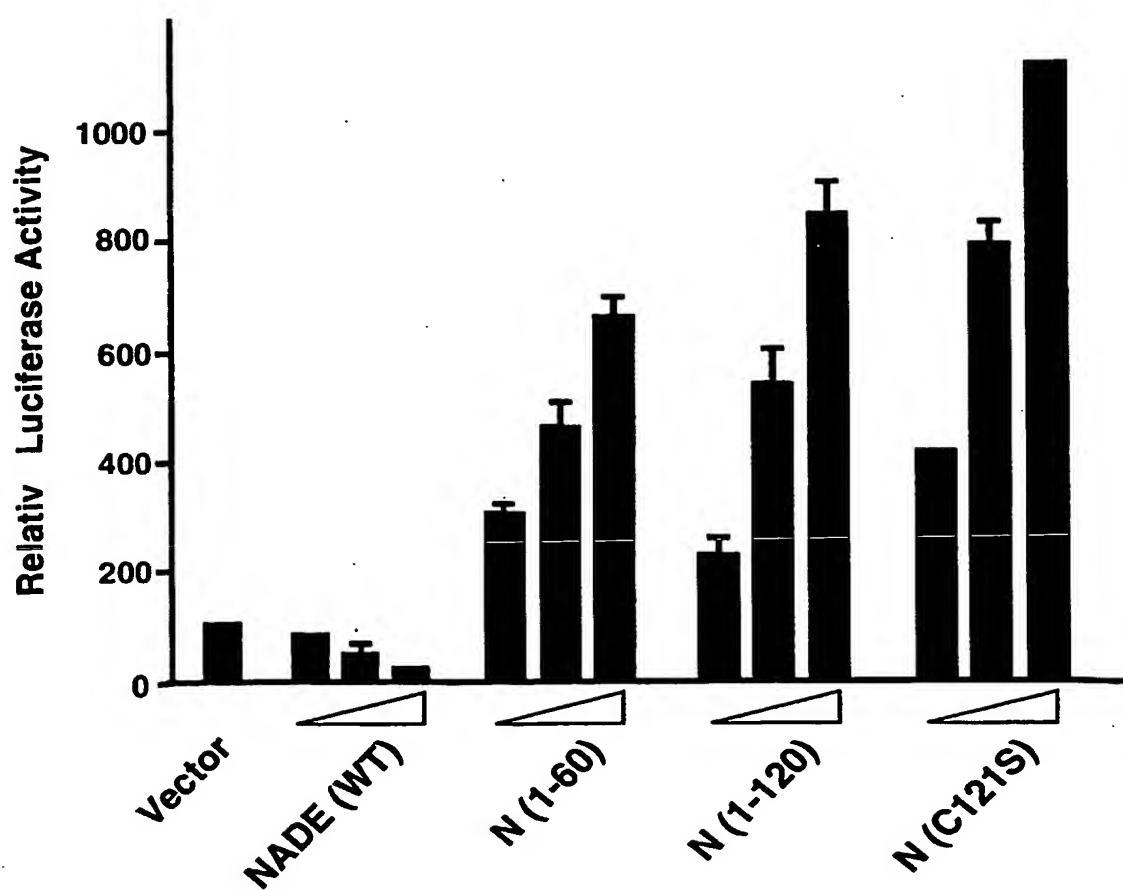


FIGURE 10

29/29

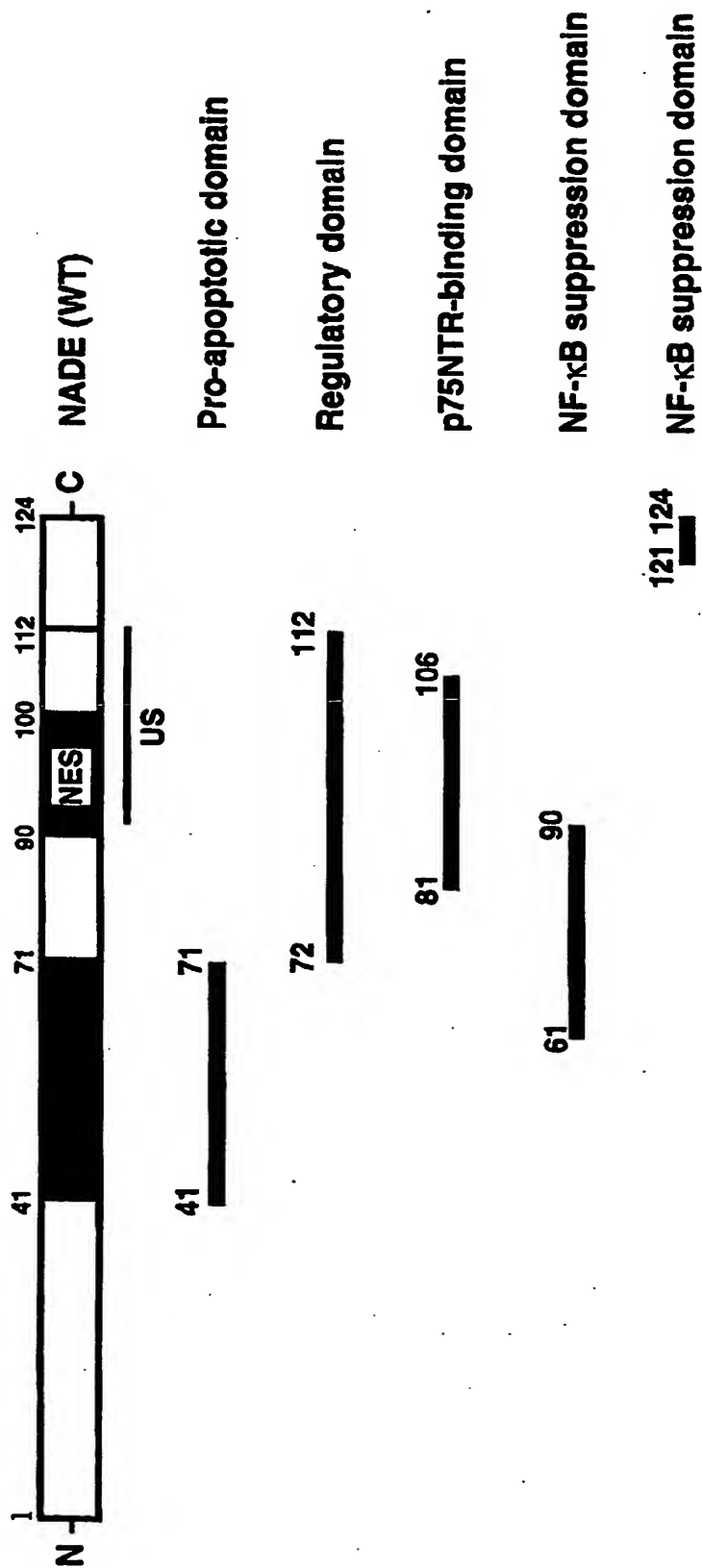


FIGURE 11